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Note to Contributors

Subscription

STUDIES ON THE DENATURATION OF LYSOZYME
I. KINETICS OF HEAT DENATURATION

By KOZO HAMAGUCHI

(*From the Department of Biology, Faculty of Science,
Uuniversity of Osaka, Osaka*)

Previously, the author studied the mechanism of surface denaturation of lysozyme (*1–4*). It was found that lysozyme is very difficult to be denatured by surface and that the properties of its monolayer are profoundly affected by whether they are spread from a solution of native lysozyme or from solutions of denatured lysozyme. As a result, we may present the surface chemical method to investigate the denaturation of lysozyme in solutions. With the aim of confirming the results obtained by surface chemical method and also of the extensive examination of lysozyme denaturation, we studied its heat denaturation.

It is well known that lysozyme is one of the most stable proteins to heat. However, quantitative investigations have not ever been carried out on its heat denaturation, except for some interesting observations on its heat inactivation (*5–7*).

In this paper will be reported the kinetics of heat denaturation of lysozyme using precipitation method as a criterion of denaturation.

MATERIALS AND METHODS

Lysozyme was prepared from hen's egg white by direct crystallization method and recrystallized four times and finally lyophilized. The molecular weight of this sample was found to be 15,100 by light scattering measurement*.

The viscosity of lysozyme solutions was measured by means of an Ostwald viscometer.

The optical rotation was determined with a Winkler Zeiss polarimeter using sodium lamp. The temperature was controlled by circulating water from a constant temperature bath through a jacketed polarimeter tube.

The precipitation method used in the present experiments was as follows: One ml. of lysozyme solution in a stoppered test-tube of about 6 ml. was heated in a bath at

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* We are indebted to Mr. T. Oyama in the Department of Chemistry for this light scattering measurement.

constant temperature.　At intervals the test-tube was taken from the bath and cooled.
When 4 ml. of borate buffer of pH 11.0 (*i.e.*, the isoelectric point of lysozyme) was added
to this solution, the denatured protein was precipitated.　The amounts of the precipitates
thus formed were measured by the micro-Kjeldahl method.

　　The pH's of lysozyme solutions were measured by a Beckman pH-meter.
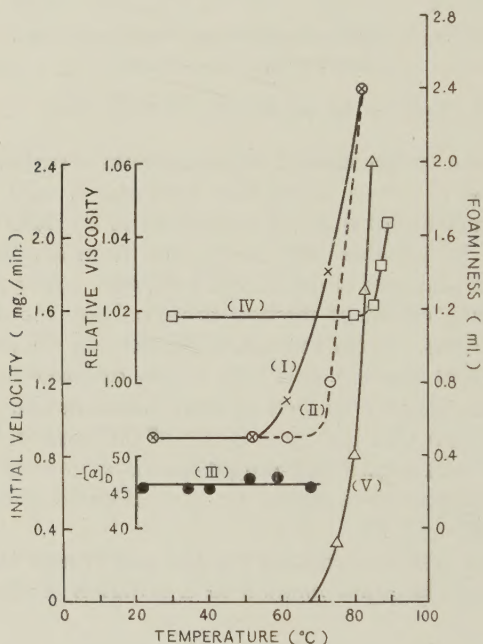
Fig. 1.　Foaminess (I and II), specific rotation (III), relative
viscosity (IV), and initial velocity (by precipitation method) (V) as a
function of temperature.　pH 5.91.

RESULTS

Comparison of Various Methods—In order to determine the method to
investigate the heat denaturation of lysozyme, foaminess, optical rotation,
viscosity, and solubility of lysozyme were measured over a wide tempera-
ture range.　The pH of the solution was adjusted to 5.91 using acetate
buffer.　The results are shown in Fig. 1.

　　In this figure foaminess represents the volume of the foam produced
by shaking 2 ml. of lysozyme solution (0.1 per cent) for ten seconds by
hand in a stoppered test-tube of about 6 ml. with an amplitude of about

30 cm. Curve (I) was obtained for the solutions immediately after heating for a quarter of an hour at each temperature and curve (II) for the solutions standing for an hour at room temperature after heating. While the foaming volume and also the foam stability of lysozyme solution were very low at the temperatures below 50°, they both increased above this temperature. The foaminess decreased, however, with time after heating the solution. This facts suggests that lysozyme might be reversibly denatured in the vicinity of 50°. It is evident from Fig. 1 that the change in foaminess appeared at the lowest temperature among the other properties.

In the measurement of optical rotation, 3.5 per cent solution of lysozyme was used (curve (III)). It was very difficult to determine the optical rotation at higher temperatures owing to the appearance of cloudiness in the visual field. However, optical rotation did not change until about 70°.

Solubility began to change from about 70°. Curve (V) shows the temperature dependence of the initial velocity (see below).

Curve (IV) shows the relative viscosities of lysozyme solutions heated for three hours at each temperature (see Fig. 2). The viscosity change occurred from 85°. Thus, the solubility of lysozyme at its isoelectric point began to change from the temperature at which viscosity did not change.

It is very difficult to investigate quantitatively the heat denaturation of lysozyme by measuring the change in foaminess (though it seems to be the most interesting phenomenon). The measurements of optical rotation and viscosity are not adequate for the examination of heat denaturation of lysozyme, because they do not reflect sensitively the change accompanying the heat denaturation. Precipitation method, therefore, is the most useful in this case.

Viscosity Measurement—Fig. 2 shows the change in relative viscosity of lysozyme solution (0.49 per cent, pH 5.91) with time. The solution after heating at 80° for 5 hours had the same viscosity as in the case of 30°. However, the precipitate was formed if the pH of this solution was adjusted to the isoelectric point. The stepwise increases of viscosity were observed above 85°. The viscosity began to increase after heating for 90 minutes and the turbidity was observed after 200 minutes.

Precipitation Method—When the pH of lysozyme solution which was heated for a desired time in a constant temperature bath was brought to its isoelectric point by adding borate buffer of pH 11.0, the precipitate was formed. The amounts of the precipitate were used as a criterion of
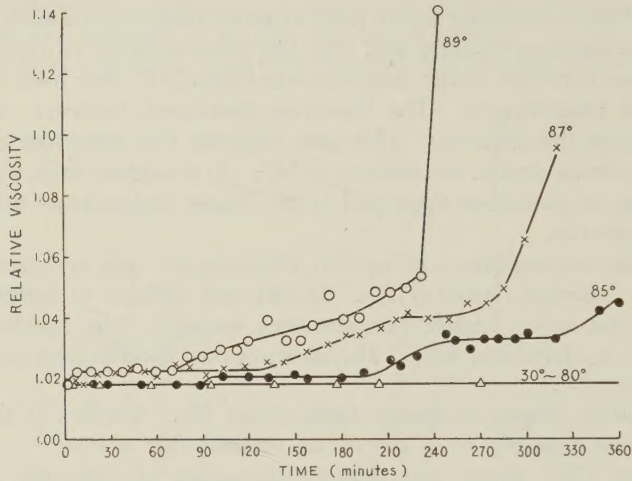
denaturation.

FIG. 3.   Relative viscosity of lysozyme solution (0.49 per cent, pH 5.91) as a function of time.
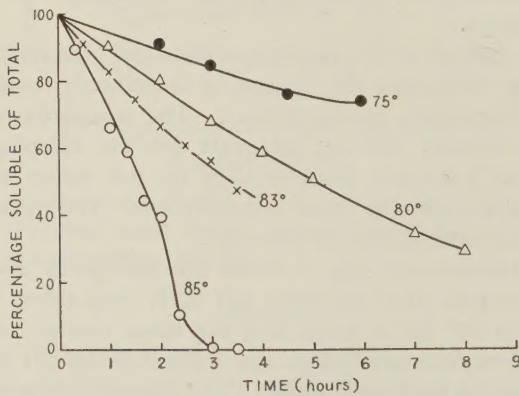
FIG. 3.   Effect of temperature on the denaturation of lysozyme (pH 5.91).   The ordinate represents per cent of soluble lysozyme.   Concentrations: 0.45 per cent at 75° and 85°; 0.48 per cent at 80° and 83°.

1.   *Effect of Temperature*—The effect of temperature on the heat denaturation of lysozyme is shown in Fig. 3.   The ordinate shows the

percentage of the soluble protein and the abscissa time in hours. The pH of the solution was 5.91.

2. *Effect of Protein Concentration*—The effect of the concentration of
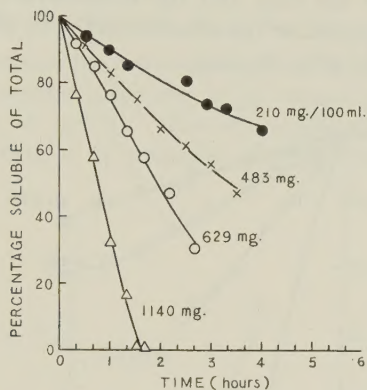
FIG. 4. Effect of lysozyme concentration. pH 5.91, temperature 83°.
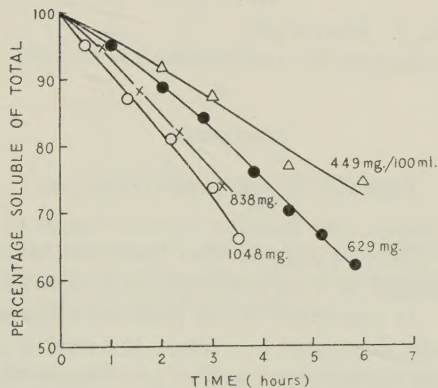
FIG. 5. Effect of lysozyme concentration. pH 5.91, temperature 75°.

lysozyme on the reaction rate at 83° and 75° was shown in Figs. 4 and 5. The pH of the solutions was 5.91. It was found from this figure that increasing the concentration of lysozyme had the effect of speeding up

the rate of its denaturation.

3. *Effect of pH's*—The heat denaturation of lysozyme depends to a greater extent on the pH's of the solution. The results are shown in Fig. 6. The pH's were adjusted with acetate buffer. The concentration of lysozyme was 0.47 per cent and the temperature was 83°. The reaction rate became greater at the alkaline pH. At pH 6.8 turbidity was observed immediately after heating.
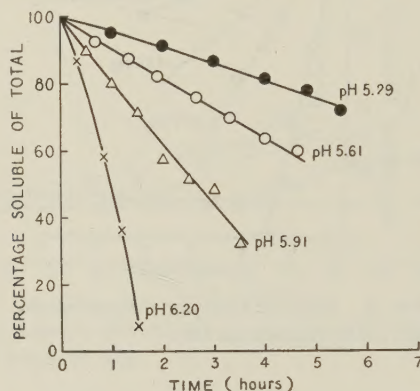
FIG. 6. Effect of pH.
Temperature 83°, concentration 0.47 per cent.

DISCUSSION

Reversible Denaturation of Lysozyme

As described above, the foaming volume began to increase rapidly from about 50°. This fact suggests that lysozyme molecules are slightly denatured and changed to a state susceptible to surface denaturation by vigorous shaking. It was found in the previous experiments that native lysozyme is not easily denatured by surface, but readily undergoes surface denaturation, if the lysozyme molecules are denatured in solutions beforehand (1). The foaming volume, however, decreased reversibly by standing at room temperature after heating. Thus, the structural change in the lysozyme molecule which affects the foaminess might be reversible. Because of the fact that the optical rotation did not change up to 70°, the reversible denaturation might not be resulted from greater change in the secondary structure. It has become clear recently that

the optical rotation reflects sensitively the change in the secondary structure of proteins or synthetic polypeptides (9–11). It is unfortunate that quantitative methods to investigate the reversible denaturation of lysozyme other than foaminess have not been established.

The Determination of the Reaction Order

The reaction order of the heat denaturation of lysozyme is determined by the differential method using the initial velocities at each initial concentration (see Figs. 4 and 5). The relationship between the concentration, c, and the rate, v, is generally given by Equation (1).

$$v = kc^n \qquad (1),$$
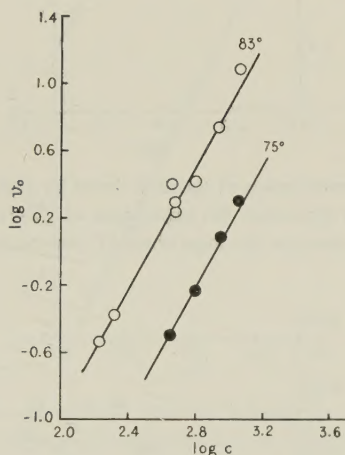
FIG. 7. Determination of reaction order by differential method. The ordinate represents the logarithms of initial velocities and the abscissa the logarithms of initial concentrations.

where n is the reaction order. In Fig. 7 are shown the relations between logarithms of the initial rates, v_o, and logarithms of the initial concentrations, c_o, at 83° and 75°. The order of the reaction determined from the slope of the straight lines shown in Fig. 7 is found to be 1.87 at both 83° and 75°.

The reaction order is also determined by half-life method. The half-life (τ) is given by the following equation:

$$\tau = \frac{1}{k} \; \frac{2^{n-1}-1}{(n-1)\; c^{n-1}} \tag{2}.$$

Therefore, the slope of the curve relating the logarithms of the half-life
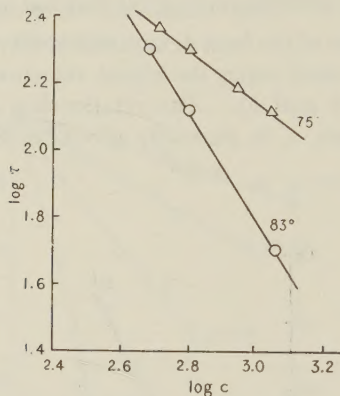
Fig. 8. Determination of reaction order by half-life method.
The ordinate represents the logarithms of 1/2- (at 83°) or 1/5-life (at 75°) and the abscissa the logarithms of concentrations.
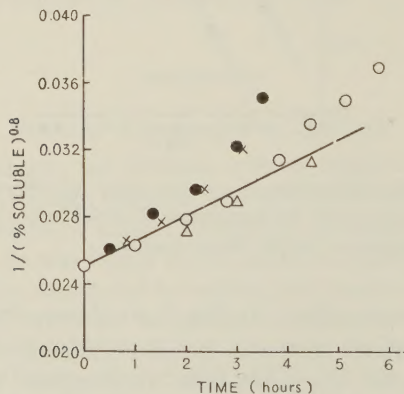
Fig. 9. Determination of reaction order by integral method.
The ordinate represents the reciprocal of (per cent soluble lysozyme)$^{0.8}$ $\triangle$, 449 mg./100 ml., $\bigcirc$, 629 mg./100 ml., $\times$, 838 mg./ 100 ml., $\bullet$, 1048 mg./100 ml. Temperature 75°.

to the logarithms of the concentration (Fig. 8) gives $(n-1)$. The order thus obtained is 2.6 at 83° and 1.8 at 75°. In the case of 75°, the time required for the concentration of the denatured lysozyme to be reduced to 20 per cent of its initial value is used instead of half-life. At 83° the order determined using the $1/5$-life is the same as that by the half-life. The order determined by half-life method is greater than by the differential method applied to the initial rates.
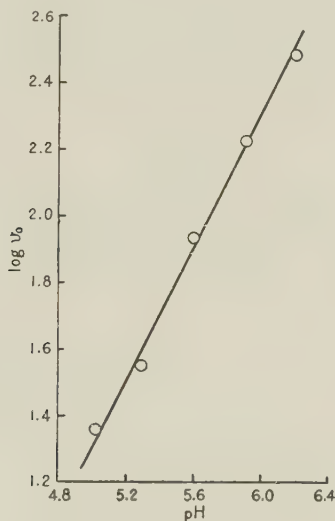
FIG. 10. The dependence of initial velocity on pH.
The ordinate represents the logarithms of initial velocities and the abscissa pH. Temperature 83°.

In the case of 75°, the reaction order is the same (about 1.8) irrespective of the method of determination. Therefore, the integration method is applied to the results at 75°. The results are shown in Fig. 9. In Fig. 9 the ordinate shows the reciprocal of (per cent soluble)$^{0.8}$. At the initial stage of the reaction the points assembles on a straight line and are independent of the initial concentration. As the reaction proceeds, however, the deviation from this line was observed and the more marked is this deviation with the increase in the protein concentration. This suggests that while the reaction of heat denaturation proceeds with the order of about 1.8 at the early stage the reactant begins to contribute to

the heat denaturation as the reaction proceeds, accompanying the gradual increase in reaction order.

The relation between the logarithms of initial velocities and pH's is obtained using the results shown in Fig. 6 (Fig. 10). The slope of the straight line shown in this figure is unity. Accordingly, the initial velocity is found to be inversely proportional to the hydrogen ion concentration.

Mechanism of Heat Denaturation of Lysozyme

From the facts described above, it is found that the following equation approximates the initial velocity of the heat denaturation of lysozyme:

$$v_o = k \frac{[P_o]^2}{[H^+]} \qquad (3),$$

where $[P_o]$ is the initial concentration of lysozyme and $[H^+]$ the hydrogen ion concentration. The constant, k, is not of a true rate constant at the denaturation step but involves the dissociation constant of ionization step or others.

The reasonable mechanism of the reaction leading to Equation (3) have not been obtained. With the heat inactivation of pepsin Casey and Laidler have found that the initial velocity was proportional to the fifth power of the enzyme concentration at lower concentrations (12). The same mechanism was not considered to be involved in the present case. However, in the case of lysozyme, the heat denaturation would be also resulted from the coöperative phenomenon, involving the participation of presumably two lysozyme molecules.

As described above, the denaturation could be detected by the precipitation method, nevertheless the viscosity did not change. The unfolding of each protein molecule should cause the increase in viscosities according to the theory of high polymer chemistry. The increases in viscosity in Fig. 2 accompanied the increase in turbidity. Therefore, the increase in viscosity is not due to the effect of each lysozyme molecule. These facts can be explained by Einstein's theory of viscosity if only the association of molecules occurred without greater loss of the native rigid structure.

Gorini and Félix (5) pointed out that the heat inactivation of lysozyme might be due to the association of lysozyme molecules and that this molecular association is inhibited by manganese ions. Quantitative comparison, however, cannot be made with their results because

the concentrations used in the inactivation experiments are much less than those of the present experiments.

Barbu and Joly (13) claimed, based on the results obtained by the measurements of flow birefringence, that the heat denaturation of serum albumin is not due to the unfolding of the molecules but to the aggregation. Kleczkowski (14) also explained the heat denaturation of serum albumin by von Smolukowski's theory of coagulation of colloidal particles. With the heat denaturation of the proteins, such as lysozyme, serum albumin, or ribonuclease, which have a number of intrapeptide disulfide bridges, aggregation rather than unfolding might be the main cause for their heat denaturation.

Because of the second order character of the heat denaturation of lysozyme the theory developed by Steinhardt (15), Levy and Benaglia (16), or Gibbs (17) which gives the pH dependence of the rate constant could not be applied.

The apparent activation energy calculated from Arrhenius equation using initial velocities at 83° and 75° was found to be 50.3 kcal./mole, which is independent of the initial concentration.

SUMMARY

The heat denaturation of lysozyme was examined by the measurements of foaminess, optical rotation, viscosity, and solubility. Especially the effects of temperature, concentration, and of pH upon the reaction rate of the heat denaturation were examined by precipitation method in detail. The results obtained were as follows:

1. The foaminess of lysozyme solution (pH 5.91) increased rapidly from about 50° with the rise of temperature. However, the change in foaminess was reversible in the vicinity of 50°. This suggests the reversible heat denaturation of lysozyme in this region. The change in the structure of lysozyme molecule which affects the foaminess seems to be minor, observing no change in optical rotation, viscosity or solubility.

2. The solubility change at its isoelectric point appeared in the temperature range in which the viscosities never change. The heat denaturation of lysozyme might be caused by the aggregation of the molecules rather than unfolding.

3. The reaction order of the heat denaturation was determined by the differential, integral, and half-life methods. The pH dependence of the reaction rate was also determined at pH's between 4.9 and 6.2. In this pH range, the initial velocity was expressed approximately by

the following equation :

$$v_o = k \frac{[\mathrm{P}_o]^2}{[\mathrm{H}^+]}$$

However, the reaction order increases as the reaction proceeds.

    4.   The apparent activation energy calculated from Arrhenius equation using the initial velocities was found to be 50.3 kcal./mole, which was independent of the initial concentration.

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BIOCHEMICAL STUDIES ON SULFATE-REDUCING BACTERIA

VIII. THE FUNCTION OF CYTOCHROME OF SULFATE-REDUCING BACTERIA IN DECOMPOSITION OF FORMATE AND REDUCTION OF SULFUR AND HYDROXYLAMINE

By MAKOTO ISHIMOTO, TATSUHIKO YAGI
AND MASARU SHIRAKI

(*From the Department of Chemistry, Faculty of Science,
University of Tokyo, Tokyo*)

Desulfovibrio cytochrome 553 or cytochrome c_3, named conveniently by P o s t g a t e (*1*), is characterized by its low potential which is adequate to strictly anaerobic character of the bacteria. In the previous paper (*2*), it was reported on the purification of the cytochrome and observation of its behavior as an intermediary electron carrier in thiosulfate reduction with hydrogen.

In the present paper, it is reported on the similar role of the cytochrome in hydroxylamine and sulfur reduction with hydrogen and in decomposition of formate to hydrogen and carbon dioxide which take place in cell-free extracts of sulfate-reducing bacteria.

MATERIALS AND METHODS

Materials—Cell-free extracts were obtained by freezing and thawing the cell suspensions, as described in the previous paper (*2*). The procedure to obtain the extracts free from cytochrome is as follows; the extracts passed through a column 1×2 cm. of ammonium or sodium salt of Amberlite IRC 50. While the cytochrome was adsorbed on the resin, the enzymes necessary for these reactions ran through the column without being adsorbed. Purified cytochrome solution was obtained by elution with $0.25\ M$ phosphate buffer, pH, 6, after washing the column with distilled water and $0.02\ M$ phosphate buffer. As the cytochrome preparation had little optical density at $280\ m\mu$, it had purity comparable to that obtained from the extracts of acetone dried cells. That sample was considered to correspond to the cytochrome fraction II from its behaviour to the resin (*2*). It was employed in many experiments.

For some experiments, cell-free extracts obtained from alumina-ground cells (*3*) were used. In this case, the extracts contained only little amount of cytochrome II owing to the adsorption on alumina.

Colloidal sulfur was prepared by acidification of sodium thiosulfate, followed by dialysis (*4*).

Methods of Assay of Enzymes—For measurements of formic hydrogenlyase activity, W a r b u r g manometers were employed under the atmosphere of nitrogen. In most cases alkali was put in the center wells to absorb carbon dioxide evolved and only hydrogen development was measured. In some experiments for the purpose of determination of carbon dioxide, alkali in the center wells of the vessels was replaced by 0.2 ml. of 5 per cent lead acetate, which absorbed only hydrogen sulfide. Amounts of carbon dioxide formed were calculated from the difference of two manometers containing alkali and lead acetate respectively.
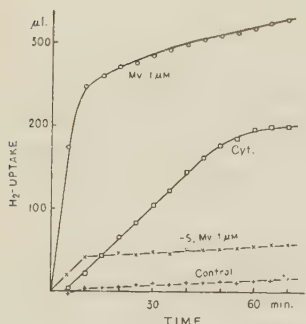
FIG. 1. Reduction of colloidal sulfur with hydrogen.

Each vessel contained 1.74 mg. N of enzyme preparation, deprived of cytochrome by passage through ion-exchange chromato-column, colloidal sulfur, 6 mg. of egg albumin, 40 μmoles of phosphate buffer, pH 7.0, and water to make the volume to 1.4 ml. Atmosphere: hydrogen. Temperature: 30°. □: with 0.023 μ atom Fe of cytochrome, ○: with 1 μmole of methyl viologen. +: without addition. ×: colloidal sulfur was replaced with supernatant after precipitation of sulfur with addition of sodium chloride to colloidal solution.

EXPERIMENTALS AND RESULTS

Reduction of Colloidal Sulfur with Hydrogen—The enzyme preparation, deprived of the cytochrome had no action for the reduction of sulfur with hydrogen. In the presence of the cytochrome as well as methyl viologen or phenosafranine, hydrogen uptake occurred (Fig. 1) Previous observation, that no hydrogen uptake occurred for the reduction of colloidal sulfur in extracts obtained by grinding cells with alumina without such dyes (*3*), was due to the lack of the cytochrome in these extracts, most part of which was eliminated by adsorption on alumina.

Reduction of sulfur with cytochrome is conceived to take place non-enzymatically, for the reduced cytochrome was oxidized by addition of colloidal sulfur even in the absence of enzyme preparation. The reaction mechanism could be indicated as follows.

$$H_2 \underset{\text{Hydrogenase}}{\xrightarrow{\hspace{2cm}}} \text{Cytochrome } c_3 \underset{\text{mon-enzymatically}}{\xrightarrow{\hspace{2cm}}} \overset{\text{S}}{\underset{\downarrow}{H_2S}}$$
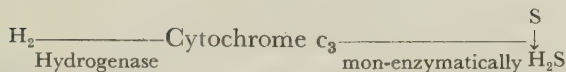
Reduction of Hydroxylamine with Hydrogen—Though hydrogen uptake due to the reduction of hydroxylamine was observed in cell-free extracts of sulfate-reducing bacteria, the extracts deprived of cytochrome did not absorb hydrogen in the presence of hydroxylamine. When methyl viologen or the cytochrome was added, the absorption of hydrogen occurred. 9.8 μ moles of hydrogen was absorbed for 10 μ moles of hydroxylamine added (Fig. 2).

Fig. 2. Reduction of hydroxylamine with hydrogen.

Each W a r b u r g vessel contained 0.65 mg. N of enzyme preparation deprived of cytochrome by passage through ion-exchange chromato-column, 10 μmoles of hydroxylamine hydrochloride, 6 mg. of ovalbumin, 60 μmoles of phosphate buffer, pH 6.4, and water to make the volume to 0.8 ml. Atmosphere: hydrogen. Temperature: 30°. $\triangle$: with 0.013 μ atom Fe of cytochrome. $\bigcirc$: with 0.1 μmole of methyl viologen. $+$: without addition.
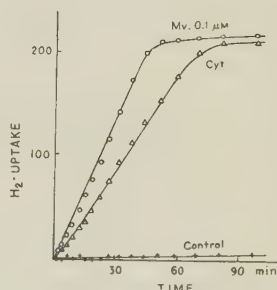
The reaction mixtures were tested for ammonia with N e s s l e r's reagent after the hydrogen uptake was ceased and found to be positive only when hydrogen uptake took place in the presence of hydroxylamine. So, it is concluded that hydroxylamine was reduced to ammonia. Only cytochrome fraction II (2) had the activity and cytochrome fraction I no effect as in the case of thiosulfate reduction. The action of the cytochrome as the electron carrier is confirmed by the fact, that in crude extracts of the bacteria, cytochrome reduced with hydrogen was rapidly oxidized by the addition of hydroxylamine.

Decomposition of Formate to Hydrogen and Carbon Dioxide—Cell-free extracts obtained by grinding cell paste with alumina, inspite of the large contents of hydrogenase and formic dehydrogenase, has little activity for decomposition of formate to hydrogen and carbon dioxide. The latter activity is greatly enhanced by addition of methyl viologen. Under such conditions, amounts of hydrogen evolved were equal to those of formate added (8.7 μ moles to 9.0 μ moles). The amounts of carbon

dioxide formed were also equal to those of hydrogen. These results indicated that the reaction took place in accordance with the following equation.

$$HCOOH = H_2 + CO_2$$

The maximum reaction rate was observed in pH range between 5 and 7.

Effects of addition of the cytochrome on the reaction were tested employing supernatant obtained by the freezing and thawing, followed by elimination of cytochrome with ion-exchange resin, as enzyme pre-
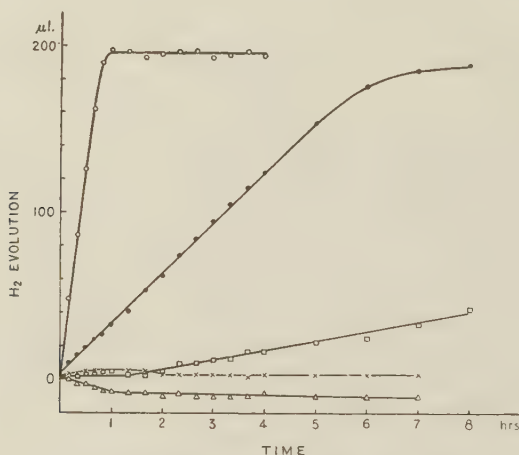
FIG. 3.   Evolution of hydrogen from formate.

Each vessel contained 0.85 mg. of enzyme preparation deprived of cytochrome by passage through ion-exchange chromato-column, 9 μmoles of formate, 123 μmoles of phosphate buffer, pH 6.2 and water to make the volume 1.7 ml. Atmosphere; nitrogen.   Center wells contained 0.2 ml. of alkali.   Temperature: 30°.   ●: with 0.043 μ atom Fe of cytochrome.   ○: with 1 μmole of methyl viologen.   ×: enzyme was previously boiled for 5 minutes.   □: without addition.   △: formate was omitted.

paration.   Though this had little activity for decomposition of formate, addition of cytochrome as well as of methyl viologen greatly stimulate the reaction (Fig. 3).

Hydrogen gas evolution from methyl viologen reduced with zinc metal (3), was observed in the presence of hydrogenase, especially vigor-

ously below pH 7, where the redoxpotential of methyl viologen is low. Recently, N. Tamiya *et al.* (5) and H. Gest (6) utilized the phenomenon to assay method of hydrogenase. The authors tried to substitute methyl viologen by cytochrome in their reaction system. The results, shown in Fig. 4, indicated the ability of cytochrome as in the case of methyl viologen to evolve hydrogen from sodium dithionite in the presence of hydrogenase. Presumably, hydrogen was evolved from the reduced cytochrome by dithionite.

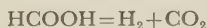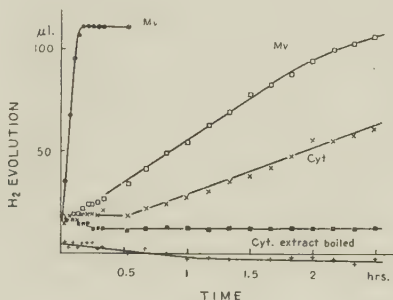Fig. 4. Evolution of hydrogen from cytochrome reduced with dithionite.

Each vessel of Warburg manometer contained 1.15 mg. N of enzyme preparation deprived of cytochrome by passage through ion-exchange chromato-column, and 80 μmoles of phosphate buffer, pH 6.8, in total volume of 2.7 ml. in main compartment and 5 mg. of solid anhydrous sodium dithionite, in side arm. Atmosphere: nitrogen. Temperature: 30°. Content of the main compartments and side arms of the vessels were mixed and the reaction was started. × : with 0.040 μ atom Fe of cytochrome. □ : with 0.01 umole of methyl viologen. ● : with 1 umole of methyl viologen. ■ : without addition. + : enzyme solution previously boiled was employed.

Evolution of hydrogen did not occur in the absence of dithionite.

DISCUSSION

From the experiments, it was shown that the cytochrome of Desulfovibrio played a role as an intermediary electron carrier in the reduction of colloidal sulfur and hydroxylamine in cell-free extracts of the bacteria. The redoxpotential of the reduction of these substances did not conflict with low potential of the cytochrome. The physiological

significance of hydroxylamine reduction in living cells is unknown and further studies are required, because it was not only resistant to reduction but also powerfully inhibitory for sulfate reduction (8).

Formate has long been known to be decomposed to hydrogen and carbon dioxide by many sorts of bacteria, but it is still uncertain, whether it is due to a single enzyme " formic hydrogenlyase," or to a multi-enzyme system containing formic dehydrogenase and hydrogenase.

The authors reported previously on the decomposition of formate to hydrogen and carbon dioxide in living cell suspension of sulfate-reducing bacteria, and succeeded to make this enzyme system cell-free. Using the cell-free extracts, it was observed that the reaction was greatly enhanced by addition of methyl viologen, in confirmation of the results of G e s t in *E. coli* (8). The observations, that the hydrogenlyase action of extracts decreased by passage through a column of cation exchange resin, to make free from cytochrome, and that the readdition of cytochrome to the extracts recovered the activity, indicate the participation of cytochrome in hydrogenlyase activity of the extracts as well as that of methyl viologen. Cytochrome and methyl viologen could be reduced with formate in the extracts and hydrogen was evolved by hydrogenase in the presence of reduced cytochrome or methyl viologen, which were continuously supplied by means of sodium dithionite. These observation indicated a role of cytochrome and methyl viologen as an electron carrier in the following reaction.

$$\text{HCOOH} \xrightarrow[\substack{\text{dehydrogenase}}]{\substack{\text{Formic}}} \quad \substack{\text{Cytochrome } c_3 \\ \text{Methyl viologen}} \quad \xrightarrow[\substack{\text{Hydrogenase}}]{} \text{H}_2$$

It is necessary to discuss on the standard redoxpotential of the cytochrome and the substrate systems. The standard potential of the cytochrome was between -0.256 (janus green) and -0.15 volt (nile blue) by authors' determination (9) and -0.205 volt at pH 7, according to the more precise measurement by P o s t g a t e (10). The value is much higher than that of formate-carbonate or hydrogen (both $E'_0 = -0.420$ volt). The standard potential of the system, thiosulfate-sulfide and sulfite, was also low (-0.423 volt according to authors' calculation from thermodynamical data (11)), though dilution from standard state elevates the potential. These facts make impossible to confirm the reaction sequence by observation of oxidation of cytochrome by these reactions which was succeeded in the case of methyl viologen.

It is surprising that a substance with such higher standard potential can substitute methyl viologen with low potential in electron transfer

between substances with much lower potential. The action of cyto-chrome may be due to its oxido-reduction with one electron change. In the case of two electron carriers, change of 0.030 volt in redoxpotential occurs for the unit change of logarithm of ratio of the oxidized type to the reduced type, but in the case of one electron carrier, change of 0.060 volt does for the same change. Accordingly, under 1 atmosphere of hydrogen, 0.05 per cent of the cytochrome exists in its oxidized form, the ratio of which is larger than that of reduced diphosphopyridine nucleotide at the normal potential of lactate-pyruvate or ethanol-acetal-dehyde. For this reason, this cytochrome can carry out its function of oxido-reduction in such a low potential region.

Though the cytochrome was found to be indispensable for the formic hydrogenlyase reaction in the extracts of sulfate-reducing bacteria, there remains possibility that factors other than cytochrome, hydrogenase and formic dehydrogenase are required, as Gest reported (8). In order to elucidate the mechanism of the reaction, it is necessary to sepa-rate enzymes and cofactors. Also the possibility is remained in some cases (in other sorts of bacteria) that the reaction is catalysed by a single enzyme.

It was anticipated in the previous report (2) that cytochrome would be found to participate in many anaerobic reactions. This paper demon-strated the role of cytochrome of sulfate-reducing bacteria in several reactions with low redox potential. Now, cytochromes need to be re-garded as leading actors not only in aerobic respiration but also in other anaerobic redox reactions.

According to Oparin (12), present aerobic living organisms evolved from anaerobes which lived on ancient earth without oxygen, and mecha-nism of aerobic metabolism developed from that of anaerobic metabolism. The facts, that cytochromes perticipate in many reactions in anaerobic metabolism may indicate, that cytochromes had been playing roles in oxido-reduction before oxygen appeared on the earth, and became com-plicated to form present systems for respiration in responce to appearance of oxygen and development of respiration.

SUMMARY

Experiments employing cell-free extracts of sulfate-reducing bacteria demonstrated that cytochrome of sulfate-reducing bacteria is an indis-pensable factor in the reduction of colloidal sulfur and hydroxylamine with hydrogen and decomposition of formate to hydrogen and carbon

dioxide.   Enzyme preparations deprived of cytochrome had little ac-
tivities to catalyze the foregoing reactions, but recovered by the addition
of the cytochrome.   The chemically reduced cytochrome with sodium
dithionite could evolved hydrogen gas in the presence of hydrogenase.
These results were fairly explicable by assuming the cytochrome as an
intermediary electron carrier in these reactions.

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ISOLATION OF α-KETOGLUTARIC-α-AMIDE AS AN INTERMEDIATE OF ENZYMATIC OXIDATION OF UROCANIC ACID

By MASAMI SUDA, YUTAKA SHIMOMURA, AKIRA KATO

(*Department of Bacteriochemistry, Research Institute for Microbial Diseases, Osaka University, Osaka*)

AND YUJIRO IMANAGA

(*Department of Chemistry, Nara Women's University, Nara*)

It has been proved recently by many investigators (*1–6*) that histidine is metabolized anaerobically to glutamic acid through various intermediates such as urocanic, formylglutamic and formamidinoglutaric acids. On the other hand, K u m a g a i (*7*) showed that oxygen uptake was observed with histidine, though not markedly, in the presence of an extract of guinea pig liver. Later, U c h i d a *et al.* (*8*) announced the partial purification of the enzyme from rabbit liver or *Pseudomonas aeruginosa* and postulated the following pathway:

$$
\begin{array}{cccccc}
\text{HC—NH} & \text{HC—NH} & \text{OC—NH} & \text{OC—NH} & \text{COOH} & \text{COOH} \\
\text{C—N} \quad \text{CH} & \text{C—N} \quad \text{CH} & \text{HC—N} \quad \text{CH} & \text{HC—NH} \quad \text{CO} & \text{CH-NH·CONH}_2 & \text{CHNH}_2 \\
\text{CH}_2 & \text{CH} & \text{CH}_2 & \text{CH}_2 & \text{CH}_2 & \text{CH}_2 \\
\text{HCNH}_2 & \text{CH} & \text{CH}_2 & \text{CH}_2 & \text{CH}_2 & \text{CH}_2 \\
\text{COGH} & \text{COOH} & \text{COOH} & \text{COOH} & \text{COOH} & \text{COOH}
\end{array}
$$

L-Histidine   Urocanic acid   Imidazolon-propionic acid   Hydantoinpropionic acid   Carbamylglutamic acid   L-Glutamic acid
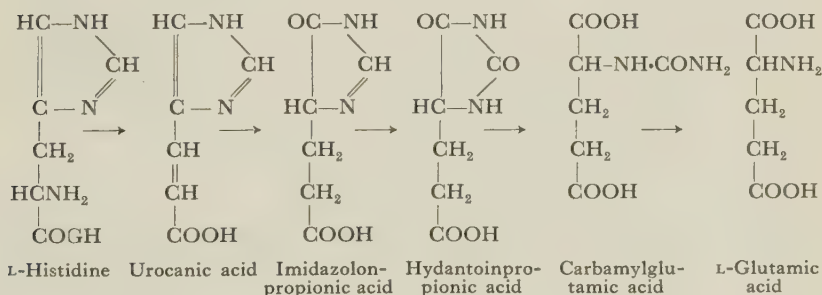
Since urocanic acid was shown to be metabolized anaerobically to glutamic acid, it became necessary to reexamine whether glutamic acid itself could be really formed aerobically from urocanic acid. Recently, I c h i h a r a *et al.* (*9–10*) reported further purification of this enzyme from the same sources and succeeded (*11*) in isolating a new intermediate, succinic monoureide, after oxidizing urocanic acid with their enzyme preparation. As reported in this paper, we also isolated a new compound, α-ketoglutaric-α-amide, as an intermediate. More recently I c h i h a r a

715

et al. (*12*) have presented evidence for the isolation of hydantion acrylic

SCHEME 1

Preparation Procedure of Enzyme

Cat Liver
| homogenized with 2 volumes of 4×10^{-2} M
| phosphate buffer, pH7.2, centrifuged

Residue
| washed with equal volume of 4×10^{-2} M
| phosphate buffer, pH 7.2, centrifuged

Residue discarded   Supernatant⟶Combined Supernatant
                                        (Extract)
                                        | adjust to pH4.8 with
                                        | 1.0 M acetate, con-
                                        | centrifuged

Residue
| washed with half volume (of the crude extract) of
| 4×10^{-2} M acetate buffer, pH4.8,

Residue discarded   Supernatant⟶Combined Supernatant
                                        adjusted to pH7.2 with
                                        1N NaOH, centrifuged

Residue discarded   Supernatant
                                        5×10^{-3} M Urocanic
                                        acid in final concen-
                                        tration added, heated
                                        at 60°–65° for 5 mi-
                                        nutes, centrifuged

Residue
| washed with half volume of the original extract of
| 4×10^{-2} M phosphate buffer, pH7.2, centrifuged

Residue discarded   Supernatant⟶Combined Supernatant
                                        (The heat-treated Enzyme)

acid after decomposing urocanic acid with an extract of acetone dried cells of *Pseudomonas aeruginosa*. They also confirmed our results of the isolation of α-ketoglutaric-α-amide under their own experimental condition.

From these data, it seems reasonable to consider that glutamic acid is not on the aerobic pathway of urocanic acid and this route is entirely different from the hydrolytic process.

EXPERIMENTAL

Preparation of the Oxidizing Enzyme—The oxidizing enzyme was separated from various animal liver as shown in Scheme 1 and their activities were compared with each other. Through these experiments, cat liver was found to be the most suitable as the enzyme source, because the oxidizing capacity of the tissue was shown to be about two or four times as active as that of the others.

It is of interest to note that the enzyme preparation does not exhibit any oxygen consumption for urocanic acid at each step of purification until the enzyme is heated at the final step. If the heat-treatment was carried out in the absence of urocanic acid, the enzyme was completely inactivated and, as shown in Fig. 1, if it was done at a temperature below 60°, no oxygen uptake was observed. From these data, it is likely
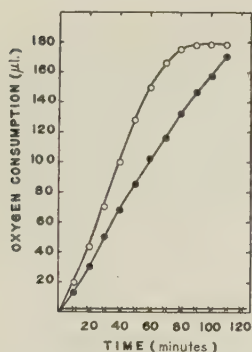
Fig. 1. Effect of heat-treatment on urocanic acid oxidizing enzyme.

The vessel contains 2.0 ml. of enzyme, 20 μM of urocanic acid, 0.5 ml. of 0.2 M phosphate buffer, pH 7.2, water to make a final volume of 3.0 ml., and 0.2 ml. of 20 per cent KOH in center well.

Atmosphere: Air

The substrate solution is tipped in after the preexisting urocanic acid is almost exhausted.

○—○; heated at 60° for 5 minutes,
●—●; heated at 65° for 5 minutes,
×—×; heated at 55° for 5 minutes.

that the preparation might be contaminated with the enzyme system concerning the hydrolytic pathway of urocanic acid which is fairly sensitive to this heat-treatment. By heating the enzyme above 70°, no activity towards urocanic acid, was observed aerobically or anaerobically.

The amounts of oxygen consumption per mole of urocanic acid varied between 0.7 to 1.5 atom with the enzyme preparations obtained. Both the reaction velocity and oxygen uptake were markedly accelerated by replacing air by oxygen as gas phase.

Isolation of α-Ketoglutaric-α-amide as a Reaction Product—Five grams of urocanic acid were incubated at 37° with continuous shaking in oxygen atmosphere with the enzyme prepared from three cat livers by the pro-

cedure described in Scheme 1. During the incubation period, several

SCHEME 2

Isolation of Reaction Product

Reaction Mixture (about 1,700 ml.)
add 1/3 volume of 10% trichloracetic acid, centrifuged

Precipitate
washed with 100 ml. of 2.5% trichloracetic acid, centrifuged

Precipitate   Supernatant——▶Combined Supernatant
adjusted to pH 7.0 with saturated Ba(OH)$_2$ solution, centrifuged

Precipitate
washed with 50 ml. of distilled water, centrifuged

Precipitate   Supernatant——▶Combined Supernatant
discarded   25% mercuric acetate in 2% acetic acid solution added until complete precipitation is insured, maintaining the pH at 6.0 with occasional addition of 1N NaOH, centrifuged

Precipitate   Supernatant discarded
washed with 0.5% mercuric acetate solution centrifuged

Precipitate   Supernatant discarded
dissolved in 2.2 liters of 20% acetic acid, centrifuged

Precipitate discarded   Supernatant
decompose with H$_2$S centrifuged

Precipitate
washed with 100 ml. of distilled water, centrifuged

Precipitate   Supernatant——▶Combined Supernatant
discarded   concentrated to about 300 ml. under reduced pressure, acetic acid eliminated with ether, treated with Amberlite IR 120 (H$^+$form), filtered

Final Filtrate

aliquots from the reaction mixture were tested for the disappearance of urocanic acid, by determining the absorption at 277 mμ. After about four hours the substrate was completely exhausted. 10 per cent trichloracetic acid solution was added to the reaction fluid and isolation procedure was carried out as shown in Scheme 2. The final filtrate was treated with a small amount of Norit, and evaporated to dryness under reduced pressure. The dried material was taken up in a small volume of water and crystallized. The material was recrystallized several times from boiling water. Finally we obtained about 450 mg. of crycrystalline compound.

Properties of the Isolated Material—The isolated compound (white, scaly crystals has a melting point at 140.5°–141.5°. V a n-S l y k e N is not detectable, but liberation of ammonia was demonstrated on boiling
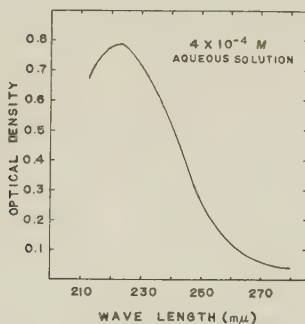
FIG. 2. Ultraviolet absorption spectrum of the isolated compound.

with NaOH. This shows the presence of amide N. Both ninhydrin and diazo reaction (in NaOH solution) are positive though the former gives yellow-brownish color, not violet as in the case of common amino acids. It decolorizes bromine and reduces potassium permanganate in sodium carbonate solution. Ultraviolet absorption spectrum shows a weak peak at 220–225 mμ as shown in Fig. 2.

Chemical Analysis of the Compound—From the value of the elementary analysis presented in Table I, the molecular formulae of the compound is considered to be $C_5H_7O_4N$. The material after hydrolysis on boiling with $3N$ HCl for 3 hours gave a 2,4-dinitrophenylhydrazone ; m.p. 210–211°. On admixture with the hydrazone of authentic specimen of α-ketoglutaric acid, the melting point of that of hydrolytic product did not show any depression. The 2,4-dinitrophenylhydrazones of both the

acid-hydrolysate and α-ketoglutarate showed the same R_f value (0.71, Solvent system; acetic acid: butanol: $H_2O=1:4:1$) by descending paperchromatography.

After decomposing the isolated compound with H_2O_2 in acetic acid medium at room temperature, succinic acid was detected by paperchromatography. From the above facts it is concluded that the chemical structure of the compound should be α-ketoglutaric-α-amide. Recently, M e i s t e r (*13*) reported the synthesis of α-ketoglutaramic acid containing γ-carboxylamide, and described this compound being deliquescent and liable to become slightly yellow on exposure to air. As thè isolated material shows none of these properties, the position of carboxylamide in our preparation should be at α-carbon, namely, α-ketoglutaric-α-amide.

Identification of the Compound by Paperchromatography—The heat-treated enzyme was fractionated with ammonium sulfate at 60 per cent saturation, then centrifuged, dissolved in small amounts of distilled water. The

TABLE I

Elementary Analysis of the Isolated Compound

	N	C	H	O	
$C_5H_7O_4N$ calculated	9.66	41.37	4.83	44.14	100%
found	10.03	42.01	5.08	42.88	

insoluble part was centrifuged off. 2.0 ml. of the enzyme was incubated aerobically in W a r b u r g vessel with 30 μM urocanic acid in the presence of phosphate buffer (pH 7.2, final concentration 0.4 M) at 37° for about five hours until the oxidation of the substrate came to an end completely. Anaerobic incubation of the same mixture was realized by routine T h u n b e r g methods. At the end of incubation, 0.2 ml. of 30 per cent TCA was added to the reaction mixture, centrifuged and the supernatants were evaporated to dryness under reduced pressure at room temperature. Each samples were dissolved in a small amounts of distilled water in order to apply to paperchromatogram which was developed with the solvent system, acetic acid; butanol; $H_2O(1:4:1)$. Only in the case of aerobic incubation, the same spot as α-ketoglutaric-α-amide, isolated as above, was able to be detected by spraying the diazoreagent, and 1 N HaOH. As for the anaerobic incubation, or the blank test (without substrate), no colored spots could be observed. From these facts, α-ketoglutaric-α-amide seems to be formed only through the oxidative pathway of urocanic acid.

DISCUSSION

Although the oxidative pathway of urocanic acid has not been established with certainty, the isolation of α-ketoglutaric-α-amide as reported above, leads us to the concept that glutamic acid does not seem to be formed aerobically.

Neither isoglutamine nor formylisoglutamine was oxidized by the heat-treated enzyme. Furthermore, after incubating them with the enzyme, the colored spot by diazo reagent could not be shown on paper-chromatogram. In the same manner, hydantoinpropionic acid itself was shown to be quite inert to the heat-treated enzyme. As mentioned above, hydantoinacrylic acid was isolated as another intermediate by I c h i h a r a *et al.* recently. So it is no wonder that the alternative pathway, hydrolytic or oxidative, should operate in the decomposition of urocanic acid. Further attempts to isolate unknown intermediates will be necessary to understand the oxidative pathway more precisely.

SUMMARY

1. The urocanic acid oxidizing enzyme was prepared from cat liver.

2. After decomposion of urocanic acid by the enzyme, α-ketoglutaric-α-amide was crystallized from the reaction fluid. This compound was shown to be formed only through the oxidative pathway of urocanic acid breakdown.

3. Some of the chemical properties of this compound were described.

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STUDIES ON CHROMOPROTEINS IN JAPANESE NORI (PORPHYRA TENERA)

## III. CHROMOPEPTIDES DERIVED FROM PHYCOERYTHRIN BY PEPTIC DIGESTION

By TERUKO FUJIWARA

(From the Laboratory of Biochemistry, Faculty of Science, Osaka University, Osaka)

L e m b e r g (*1*) and S i e d e l (*2*) were the first to show that the prosthetic group of phycoerythrin is composed of a bile pigment mesobilirhodin. They have also pointed out that the prosthetic group is much

Diagram 1
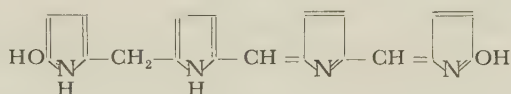
Mesobilirhodin

more firmly bound to the protein moiety than in the case of hemoglobin and that it is impossible to split the linkage by mild procedures. L e m b e r g (*1*) and K i t a z a t o (*3*) have, therefore, employed pepsin to effect the cleavage and thus succeeded in isolating a purple pigment from phycoerythrin. This pigment called "phycoerythrobilin" by L e m b e r g was shown to contain some peptide-like materials and does not appear to be completely pure.

The present investigation was undertaken with a purpose of obtaining pure chromopeptides from phycoerythrin and thus elucidating the chemical structure around the chromophore group of this pigment. To this end, crystalline phycoerythrin prepared from Japanese Nori, *Porphyra tenera*, was subjected to partial peptic digestion and the resulting chromopeptide fractions were extracted with isoamylalcohol.

Several methods were then tried to fractionate these chromopeptides. Since T u p p y and P a l é u s (*4*) succeeded in purifing a hemopeptide from peptic digests of cytochrome c with the aid of a celite column, the same

technique was first applied to the chromopeptide mixture from phy-coerythrin. Only two colored fractions, *viz.*, purple and violet, were, however, obtained by chromatography with celite as well as with starch columns. The mixture was then separated into five colored bands, *viz.*, three purple and two violet ones, by paper electrophoresis using lutidine-acetate buffer (pH 5.0–6.0) as solvent. The most satisfactory separation of chromopeptides was, however, achieved by means of ion exchange chromatography on Amberlite IRC-50 column. This method permitted the separation of the mixture into 16 fractions. It has thus been established that what was called " phycoerythrobilin " by L e m-b e r g is composed of at least 16 chromopeptide fractions.

The main fraction, referred to as C_1, exhibited a violet color in acidic solution and seemed to be more stable than the other peptides which turned brown on exposure to atmospheric oxygen. The amount of C_1 peptide was estimated to be one seventh of the total chromopeptide fractions and one thirtieth of the original protein. With the aid of the S a n g e r's method, it was found that the N-terminal amino acid of this peptide is phenylalanine. Fifteen amino acids were detected in this peptide ; aspartic acid, glutamic acid, glycine, alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, threonine, serine, arginine, lysine, histidine and cystine. Proline and methionine were missing in the peptide, although these two amino acids are present in the original pro-tein. Assuming one mole of phenylalanine per peptide, it is calculated that the peptide contains 43 to 44 amino acid residues.

EXPERIMENTAL AND RESULTS

Materals—Phycoerythrin used in this experiment was prepared by the procedure described previously (*6*) from the air-dried Japanese Nori *Prophyra tenera* and recrystallized three times. The pepsin used was a twice crystallized commercial Worthington sample.

Peptic Digestion of Phycoerythrin—The peptic digestion was carried out at 37°, in HCl solution of pH 1.6. In all experiments, the substrate con-centration was about 0.2 per cent. The rate of hydrolysis was measured at an enzyme-substrate concentration ratios 1/7 and 1/30 and the course of digestion was followed both by the ninhydrin procedure (*7*) and by the estimation of the absorption at 495 mμ in the trichloroacetic acid filtrate of the reaction mixture which is the characteristic absorption maximum of the chromopeptides (see Figs. 2 and 5). The course of hydrolysis is shown in Fig. 1.

For the chromatographic experiments, 220 ml. of the aqueous solu-
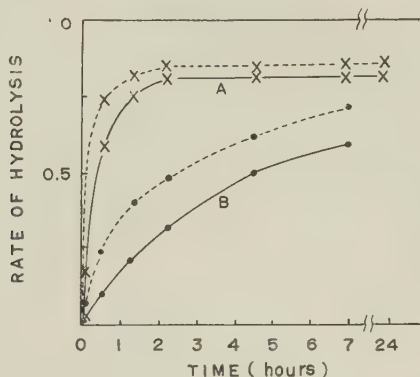
Fig. 1.   The rate of hydrolysis of phycoerythrin by pepsin at 37°.
- - - - - Ninhydrin procedure, ——·—— Absorption spectra
of 495 mμ

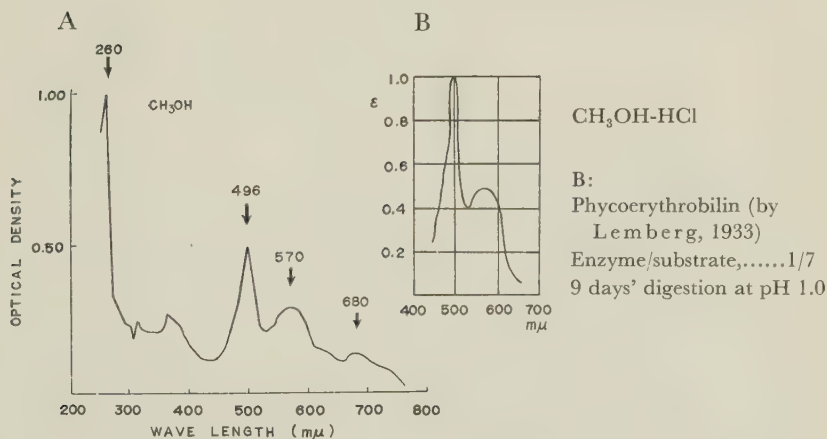A; Enzyme-substrate, 1/7,   B; Enzyme-substrate, 1/30

Fig. 2.   The absorption spectra of amylalcohol fraction extracted
from peptic digestion mixture.
A: (by the author)
Enzyme/substrate......1/7, at 37°, 2 hours' digestion at pH 1.6

tion containing 467 mg. of phycoerythrin was added with 24 ml. of 0.1 N

hydrochloric acid, and the mixture was brought to pH 1.6. To the solution was added 65 mg. of pepsin dissolved in 40 ml. of dilute hydrochloric acid (pH 1.6). After 2 hours, the reaction was stopped by adding the same volume of 20 per cent of trichloroacetic acid and the precipitate was removed by centrifugation. The supernatant liquid was extracted with isoamylalcohol. The extract was added with a seven-fold volume of petroleum ether and allowed to stand overnight. The lower phase containing chrmopeptides was separated and then dried in vacuo in an atmosphere of nitrogen below 40°. The residue was dissolved in methanol and the peptone-like materials were removed by centrifugation. These procedures were repeated three times. Finally the methanol extract was dried up as described above. The absorption spectrum of the final product is shown in Fig. 2-A. This spectrum is in good agreement with the result of Lemberg as shown in Fig. 2-B. Yield: 120 mg.

Chromatography of Chromopeptides on Amberlite IRC-50 Column—The chromatographic separation was carried out with a 60×1.5 cm. column of Amberlite IRC-50 (H-form, 150–300 mesh). The solvent systems employed were as follows:

a) Methylethylketone :Acetone: H_2O 15:15:70
b)   ,,   : ,,   :pH 3.0 Acetic acid solution   15:15:70
c)   ,,   : ,,   :pH 2.5 HCl solution   15:15:70
d)   ,,   : ,,   :pH 2.0 HCl solution   15:15:70
e)   ,,   : ,,   :pH 1.0 HCl solution   20:20:60

The effluent was distributed into 270 tubes, each receiving 2.0 ml. thereof. The initial flow rate was about 8 ml. per hour. The elution curves were obtained by the determination of the absorption at 495 mμ and by the ninhydrin method (7) for which was employed one-tenth of each fraction. The curves obtained by the ninhydrin method and by the spectrophotometoric procedure exhibited approximately the same peaks. The elution curves are shown in Fig. 3. Each peptide preparation was collected by removing the solvents in vacuo from the corresponding fractions and used in the following experiments.

Paper Chromatography of Chromopeptides—Chromopeptide fractions obtained by the Amberlite IRC-50 column were hydrolyzed with 6 N HCl at 105° for 24 hours and examined by two dimentional paper chromatography using the solvent systems of 80 per cent phenol-NH_3 and buthanol-acetic acid-water (4:1:1). The results are shown Table I.

Amino Acid Analysis of Main Peptide C_1—For the quantitative determi-

nation 8 mg. of C_1 peptide were hydrolyzed in a sealed tube with 6 N HCl at 105° for 24 hours and the hydrolysate analyzed by the M o o r e - S t e i n method (7–9), as in the case of phycoerythrin (10). Cystine was estimated as cysteic acid after performic acid oxidation. The results are summarized in Table II. Fifteen amino acids, aspartic acid, glutamic acid, glycine, alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, threonine, serine, arginine, lysine, histidine and cystine were found.
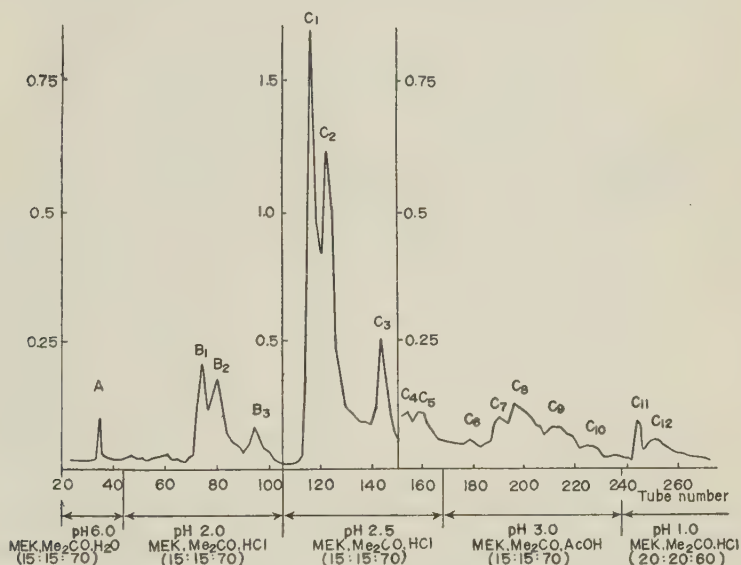
Fig. 3.   The elution curves of chromopeptide of phycoerythrin by IRC-50 ion exchange chromatography.   (Column 0.9×60 cm.)

The contents of aspartic acid, alanine and lysine were estimated to be 7,6 and 4 moles, respectively, per peptide chain. However, as shown in Table II, the aspartic acid content measured by paper chromatography of the DNP derivatives (11, 12) was found to be only 4 moles per peptide chain.   Cause for this discrepancy is not clear.

N-Terminal Group of C_1 Peptide—C_1 peptide (2.34 mg.) was dinitrophenylated by the S a n g e r's method (5) and the excess dinitrofluorobenzene was removed with ether.   The residue was acidified. and extracted with ethylacetate.   The green DNP-chromopeptide was thereby

transferred into the ethyl acetate layer. The DNP-peptide was then obtained from the extract by evaporating the solvent. Furthermore, C_1 peptide (2.6 mg.) was treated with performic acid (13), and then dinitrophenylated using trimethylamine by Sanger and Thompson method (14). The DNP-chromopeptides obtained by the above two methods were hydrolyzed with 6 N HCl at 105° for 2, 6 and 24 hours, respectively. The N-terminal group of C_1 peptide was obtained as DNP-phenylalanine by the paper chromatography (11, 12).

TABLE I

Amino Acid of Peptides Obtained by Peptic Digestion

Peptides No.	Amino acids														Yield from 467 mg. of proteins (mg.)	Color
	Cys	Asp	Glu	Ser	Thr	Ala	Val	Leu	Phe	Tyr	Gly	Arg	Lys	His		
A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	54.31	Yellow
B_1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	3.07	Redish yellow
B_2	+	+	+	+	+	+	+	+	+	+	+		+	+	2.57	Redish brown
B_3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1.82	,,
C_1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	16.56	Violet
C_2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	9.26	,,
C_3	+	+	+	+	+	+	+	+	+	+	+		+	+	3.11	,,
C_4	+	+	+			+	+	+	+	+	+				1.18	,,
C_5	+	+	+		+	+	+	+	+	+	+		+	+	1.57	Purple
C_6	+	+	+					+							trace	Brown
C_8	+	+	+	+		+	+	+	+	+	+	+	+	?	1.51	,,
C_{10}	+	+	+	+	+	+	+	+	+	+	+	+	+	?	6.51	Brown
C_{11}	+	+	+	+	+	+	+	+	+	+	+	+	+	?	2.50	Yellowish brown
C_{12}	+	+	+	+	+	+	+	+	+	+	+	+	+	?	5.94	Redish brown

C-Terminal Group of C_1 Peptide—C-terminal amino acid was determined by hydrazinolysis (15). 0.5 ml. of anhydrous hydrazine, was added to 2.5 mg. of C_1 peptides and the mixture was heated at 100° for 10 hours followed by the treatment with isovaleraldehyde and then immediately dinitrophenylated. DNP-derivatives were paper-chromatographed as described above. A DNP-derivative was obtained and identified as DNP-bilirubin by paper chromatography (Fig. 6) (11, 12).

DISCUSSION

According to Lemberg (*1*) and Kitazato (*3*) it has been pointed out that "phycoerythrobilin" obtained from phycoerythrin by peptic digestion at pH 1.0 for 9 days, is soluble in water and alconol, insoluble in ether and chloroform, and gives positive biuret reaction while Gmelin reaction is negative. The absorption spectra in $CHCl_3$–HCl solution show maxima at 495 and 575 mμ (Fig. 2-B).

In the present work, the chromopeptide fractions corresponding to "phycoerythrobilin" obtained by 2 hours' peptic digestion at pH 1.6

TABLE II

Amino Acid Composition of C_1 Peptide

Amino acid	Moles of amino acid		Amino acid residues per Mole of peptide	
Aspartic acid	6.75	(4.21)*	7	(4)*
Glutamic acid	2.02		2	
Serine	2.48		2~3	
Threonine	2.14	(0.95)*	2	(1)*
Glycine	2.53		3	
Alanine	5.95		6	
Valine	2.67		3	
Leucine	1.68		2	
Isoleucine	2.74		3	
Tyrosine	1.83		2	
Phenylalanine	1.00		1	
Histidine	2.12		2	
Lysine	3.89		4	
Arginine	3.01		3	
$1/_2$ Gystine	1.09		1	
Totals			43~44	

* The values of DNP method by paperchromatography.

were also found to have the same properties as cited above. The absorption maxima of the peptic digestion products were observed at 496, 570 and 680 mμ. As compared with the original protein, the characteristic absorption maxima at 546 and 560 mμ are almost lost. After peptic digestions, the absorption at 496 mμ due to tetrapyrrole or dipyrrole structures (*16*) becomes conspicuous.

It is, therefore, suggested that the chromopeptides obtained above contain a bile pigment which has suffered some modifications in the structure of chromophore group of the original phycoerythrin. As

shown in Fig. 6, the intense absorption at 496 mμ can be seen in the spectra of all peptide fractions, A, B and C ; but it is considerably weak in the C_{10}, C_{11} and C_{12} fractions. The main C_1 peptide exhibits an ab-
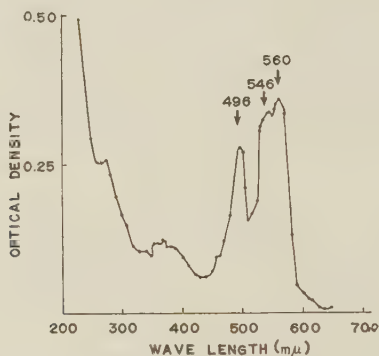

FIG. 4. Absorption spectrum of physoerythrin (pH 6.8).
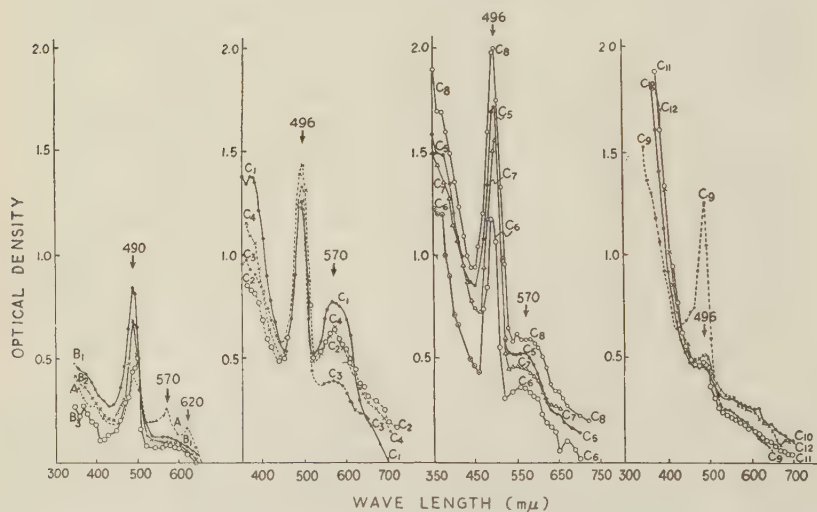

FIG. 5. Absorption spectra of chromopeptides derived from phycoerythrin.

sorption at 570 mμ in addition to the intense maximum at 496 mμ.

It has been pointed out by Lemberg (17) and Fischer (18) that biliverdin dimethylester gives a negative Gmelin reaction. Since

our chromopeptides do not give Gmelin reaction, it seems likely that the prosthetic group (mesobilirhodin) does not possess any free hydroxyl groups in its terminal pyrroles.

The qualitative amino acid analysis shows that all chromopeptide fractions obtained by peptic digestion possess similer amino acid patterns except for the C_4 to C_6 peptide in which no histidine, arginine and lysine could be detected by paper chromatography. It appears from the results of amino acid analysis that C_1 peptide is rather a large peptide composed of fifteen different amino acids. Only two amino acids, proline and methionine, are missing in this peptide as compared with the original protein. These findings are in accord with the previous reports (19, 20) that hydrolysis of protein by pepsin generally produce larger peptides.
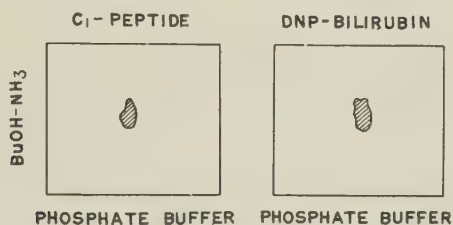

FIG. 6. C-terminal group of peptide by paper chromatography.

The N-terminal amino acid of C_1 peptide was identified as phenylalanine. In this determination most of terminal DNP-phenylalanine were released from the peptide after 24 hours hydrolysis. This suggests the possible presence of a peptide bond resisting hydrolysis in N-terminal sequence of the C_1 peptide.

By hydrazinolysis and subsequent dinitrophenylation of phycoerythrin, the author was able to obtain a DNP-derivative which behaved as DNP-bilirubin on the paper chromatogram (Fig. 6). When the same procedures were applied to C_1 peptide, a DNP-derivative was obtained and again identified as DNP-bilirubin. No DNP-amino acids were, however, detected in these procedures. One possibility is that asparagine or glutamine may occupy the C-terminals of the peptide ; both aspartic and glutamic acids are abundant in the peptide (7 and 2 moles, respectively) and if these amino acid amides reside in the C-terminal position, then detection by hydrazinolysis will be very difficult.

T h e o r e l l (21) and T u p p y et al. (4) reported that the prosthetic group of cytochrome c is linked to the protein moiety by thioether linkages

involving cysteine residue and this is the reason why the prosthetic group cannot be split by acid hydrolysis. It seems, therefore, of interest to investigate the distribution of sulfur-containing amino acids in the chromopeptide fractions. The amino acid analysis show that cystine is present in all the chromopeptides obtained and the C_1 peptide contains one half mole of cystine. It has been noted in an previous investigation (10) that phycoerythrin contains excess sulfur which could not be accounted for as cystine, cysteine and methionine. Thus it seems probable that these excess sulfurs in phycoerythrin may be concerned in the linkage with prosthetic bile pigment as thioether bridges as in cytochrome c. However, much work on the content of sulfur and its form in the C_1 peptide, should be done before decisive conclusions can be drawn on these points.

SUMMARY

The chromopeptides obtained from phycoerythrin by 2 hours' peptic digestion were found to be larger peptides, and could be separated into 16 peptide fractions.

The main chromopeptide C_1 contains 16 amino acids with phenylalanine as N-terminal amino acid. Assuming that the C_1 peptide has 1 mole of phenylalanine per mole of peptide, it is assessed as composed of 43 or 44 amino acid residues.

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BACTERIAL METABOLISM OF γ-AMINOBUTYRIC ACID*

II. CATABOLIC PATHWAY OF γ-AMINOBUTYRIC ACID IN ACHROMOBACTER**

By TANEAKI HIGASHI, TAKEKAZU HORIO
AND KAZUO OKUNUKI

(From the Department of Biology, Faculty of Science, University of Osaka, Osaka)

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γ-Aminobutyric acid is well known as a decarboxylated product of glutamic acid by several kinds of tissues and bacteria, and has been supposed as an intermediate in the catabolism of putrescine (*1*), but the catabolic pathway of γ-aminobutyric acid in the living cells was little known (*2, 3*). Recently an enzyme has been demonstrated in mammalian tissues, which catalyzes the transamination of γ-aminobutyric acid or β-alanine with α-ketoglutaric acid (*4, 5*). These reactions have also been observed with preparations of certain microörganisms (*6*).

This report deals with studies on some aspects of catabolism of γ-aminobutyric acid in the living cells of *Achromobacter*.

MATERIALS AND METHODS

Bacterial Strain—Achromobacter UH₃

Culture Conditions and Preparation of Cell Suspension—The procedures were the same as previously reported (*7, 8*) except that the suspension was prepared by suspending the cells in distilled water instead of the buffer used in the previous experiments.

Preparation of the Cells Adapted to a Substrate—Using a flask, the cells were shaked at 30° in phosphate buffer solution (pH 7.0, $M/50$) containing a substrate ($M/100$). At the same time, the oxidation of the substrate by the cells was manometrically measured in the same component. When the cells was completely adapted to the substrate and became to oxidize it in a maximum rate, the content of the flask was centrifuged. The precipitated cells were washed three times with distilled water and suspended again in

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distilled water.

Preparation of Cell-free Enzyme—The cells were ground with dry ice into a frozen powder and thawed at 30°. This procedure was repeated again and the same weight of phosphate buffer solution (pH 6.3, $M/10$) was added to the paste of the treated cells. After being kept in a refrigerator overnight, the suspension was centrifuged at 14,000 r.p.m. for twenty minutes. The supernatant was used as the enzyme solution.

Manometric Measurement—Refer to the previous report (8).

Buffer Solution—In most of the cases, phosphate buffer solution was used which consisted of Na_2HPO_4 and NaH_2PO_4. In the other cases, tris(hydroxymethyl)amino-methane-hydrochloride buffer solution was used.

<center>RESULTS</center>

Oxidations of Succinic, Fumaric, and Malic Acid by the Cell Suspension— *Achromobacter UH_3* adaptively oxidized γ-aminobutyric acid. The previous investigation by O k u n u k i *et al.* suggested that γ-aminobutyric acid might be aerobically catabolized *via* succinic acid by this microörganism (7).

<center>TABLE   I</center>

<center>*Oxidation of Succinate by the Cell Suspension of Achromobacter UH_3*</center>

The content of the manometer vessel: Cell suspension 1.0 ml., $M/10$ phosphate buffer solution (pH 7.0) 0.4 ml., $M/100$ succinate 0.4 ml., and water to bring the total volume to 2.0 ml. In the centre well, 20 per cent KOH 0.2 ml. The final concentration of streptomycin was 20 μg/ml. Reaction temperature, 30°.

Kind of cells	Oxidation of succinate by the cell suspension	
	Oxygen uptake for initial 15 minutes (μl)	Inhibition by streptomycin (%)
Non-treated cells	14	45
Cells adapted to γ-aminobutyrate	30	0

The semiadaptive oxidation of succinic acid by the cell suspension suffered 45 per cent inhibition in the presence of 20 μg./ml. of strepto-mycin. However, the cells previously adapted to γ-aminobutyric acid could rapidly oxidize succinic acid without receiving streptomycin in-hibition (Table I). The same results were obtained with fumaric and malic acids. Therefore, it seems likely that γ-aminobutyric acid was catabolized *via* succinic, fumaric and malic acids, since the cells previously

adapted to the former acid were " successively adapted " (9) to the latter three acids.

Stimulation of the γ-Aminobutyric Acid Oxidation of the Cell Suspension by Addition of α-Keto Acids—The γ-aminobutyric acid oxidation of the cell suspension was stimulated by additions of α-ketoglutaric acid and oxaloacetic acid but not by addition of pyruvic acid (Table II). This phenomenon suggests that the transamination of γ-aminobutyric acid takes place with the former two α-keto acids in the living cells.

γ-Aminobutyric Acid Oxidation of β-Alanine-Adapted Cells—The cells previously adapted to β-alanine oxidized γ-aminobutyric acid far more rapidly

TABLE II

Stimulation by α-Keto Acids of γ-Aminobutyrate Oxidation by the Cell Suspension

Concentration of each substrate, 4 μM. The other experimental conditions were the same as for Table I.

Substrate	Oxygen uptake for initial 30 minutes (μl)		
	Expt. I	Expt. II	Expt. III
γ-Aminobutyrate	35	33	35
α-Ketoglutarate	80		
Oxaloacetate		55	
Pyruvate			47
γ-Aminobutyrate + α-Ketoglutarate	220		
γ-Aminobutyrate + Oxaloacetate		130	
γ-Aminobutyrate + Pyruvate			72

than the non-adapted cells, though a little more slowly than the cells adapted to γ-aminobutyric acid itself (Table III). However, the oxidation was no longer affected by the presence of streptomycin as well as in the case of the cells previously adapted to γ-aminobutyric acid. Though the cell suspension adaptively oxidized α-alanine as fast as β-alanine and more slowly δ-aminovaleric acid, adaptation of the cells to them caused neither stimulation of the oxidation of γ-aminobutyric acid nor removal of the streptomycin inhibition.

Oxidation of γ-Aminobutyric Acid by the Cell-free Extract—The cell-free extract of the microörganism catalyzed the oxidation of glutamic acid in the presence of diphosphopyridine nucleotide (DPN). Under the same

experimental conditions, the extract could not decompose γ-aminobutyric acid and α-ketoglutaric acid added as a sole substrate, but when γ-amino-butyric acid and α-ketoglutaric acid were added together to the extract, oxygen was consumed along a curve of the same incline as in the oxidation of glutamic acid (Fig. 1).

On the other hand, the cell-free extract from the cells previously adapted to γ-aminobutyric acid was able to oxidize γ-aminobutyric acid without addition of α-ketoglutaric acid, and oxygen consumption increased in parallel with the amount of the enzyme solution added but not with the moles of the substrate added : When the enzyme solution was added two and three times much in amount, the oxygen consump-

TABLE III

Effect of Streptomycin on the γ-Aminobutyrate Oxidation by the Cell Suspension

The experimental conditions were the same as for Table I, except that 4 μM of γ-aminobutyrate was used as the substrate. The micro-örganism adapted previously to succinate, α-ketoglutarate, or acetate could also oxidize γ-aminobutyrate more rapidly than the non-treated one and streptomycin gave less influence.

Kind of cells	Oxygen uptake for initial 15 minutes (μl)	Inhibition by streptomycin ($\%$)
Non-treated	10	44
Adapted to γ-aminobutyrate	65	0
Adapted to β-alanine	38	0
Adapted to δ-aminovalerate	11	43
Adapted to α-alanine	11	19

tion increased approximately two and three times, respectively, irrespective of the preincubation time during which the enzyme solution and the substrate were separately incubated. These facts indicates that the cell-free extract from the cells previously adapted to γ-aminobutyric acid contained some substance which was not contained in the non-adapted cell-free extract. In fact, after being dialyzed, the enzyme solution failed to oxidize γ-aminobutyric acid without addition of α-ketoglutaric acid, like the extract from the non-adapted cells (Fig. 2). The reaction was almost completely suppressed in the presence of semicarbazide (Fig. 3). It seems likely that semicarbazide trapped a certain keto acid
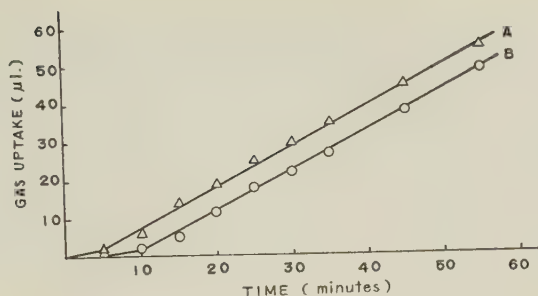
FIG. 1. The oxidation of glutamate or of γ-aminobutyrate and α-ketoglutarate by the cell-free extract from the normal cells.

The content of manometer vessel: Cell-free extract 1.0 ml., $M/5$ phosphate buffer solution (pH 8.0) 0.5 ml., DPN (approximately 92 per cent in purity) 1 mg., each substrate 20 μM, methylene blue $M/10000$ in final concentration, and water to bring the total volume to 2.0 ml. In the centre well, 20 per cent KOH 0.2 ml. The reaction temperature, 30°.

Curve A, glutamate; B, γ-aminobutyrate + α-ketoglutarate. Under the same conditions, this extract did not oxidize γ-aminobutyrate and α-ketoglutarate, when the two acids were separately added.

FIG. 2. Oxidation of γ-aminobytrate by the cell-free extract from the γ-aminobutyric acid-adapted cells.

The experimental conditions were the same as for Fig. 1, except that the total volume was adjusted to 4.0 ml.

Curve A, the cell-free extract 1.0 ml. + γ-aminobutyrate; B, the extract 2.0 ml. + γ-aminobutyrate; C, the extract 3.0 ml. + γ-aminobutyrate; D, dialyzed extract (the volume increased by dialysis against $M/20$ phosphate buffer solution, approximately 30 per cent) 3.0 ml. + γ-aminobutyrate; E, dialyzed extract 2.0 ml. + γ-aminobytyrate + α-ketoglutarate.

involved in the enzyme solution which could be utilized only in the co-existence with γ-aminobutyric acid. The enzyme solution had succinic oxidase and strong oxaloacetic decarboxylase activities but did not attack pyruvic, malic, fumaric, and α-ketoglutaric acid. The enzyme solution did not consume oxygen when pyruvic acid and γ-aminobutyric acid were added together, in accordance with the case of the living cells (Table II). The oxidation of γ-aminobutyric acid by the enzyme solution was hardly observed in tris(hydroxymethyl)aminomethane-hydro-chloride buffer solution used instead of phosphate buffer solution (Fig.

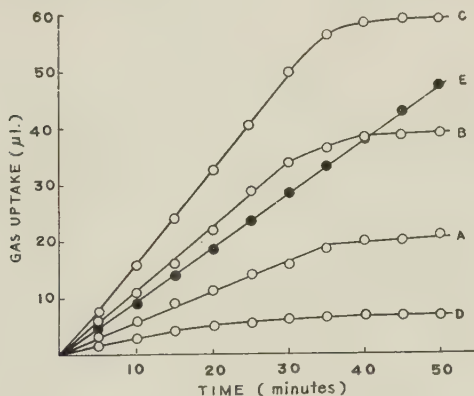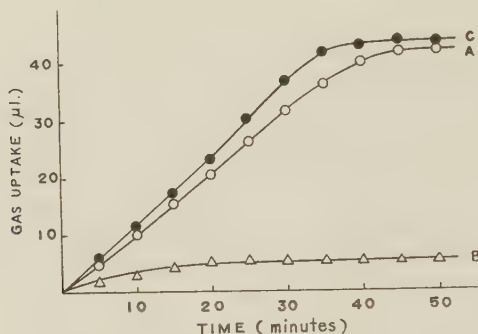Fig. 3. Effect of semicarbazide and manganese on the γ-amino-butyrate oxidation by the cell-free extract from the γ-aminobutyric acid-adapted cells.

The experimental conditions were the same as for Fig. 1, except that the total volume was adjusted to 4.0 ml. In all cases, 2.0 ml. of the extract was used.

Curve A, γ-aminobutyrate; B, γ-aminobutyrate + semicarbazide 10 μM; C, γ-aminobutyrate + $MnSO_4$ 1 μM. With $MgSO_4$, result similar to $MnSO_4$ was obtained.

4). These facts led us to a consideration that the enzyme solution extracted from adapted cells contained at least α-ketoglutaric acid, γ-amino-butyric-α-ketoglutaric transaminase and glutamic dehydrogenase. In fact, γ-aminobutyric acid oxidation of the cell-free extract in the presence of α-ketoglutaric acid proceeded in almost the same rate independently of the treatment for adaptation. The α-ketoglutaric acid, derived by enzymic deamination from glutamic acid which, in turn, was produced by γ-aminobutyric-α-ketoglutaric transaminase system, might be decomposed by the hydrogen peroxide which was formed via the methylene

blue used as a hydrogen carrier, since the decomposition of the acid was manometrically demonstrable by the addition of hydrogen peroxide.

Effect of pH and of Metal Ion to γ-Aminobutyric Acid Oxidation by the Cell-free Extract—The reaction proceeded very slowly at lower pH range. The optimal pH was approximately 8. Mg^{++} and Mn^{++} gave a little stimulatory effect on the reaction rate (Fig. 3).

DISCUSSION

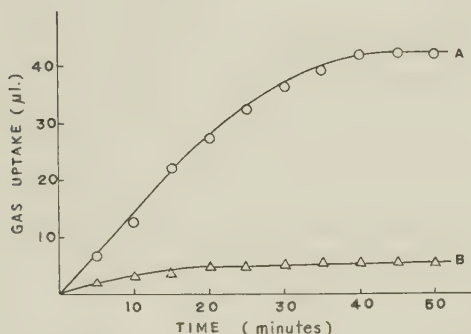The cell suspension of *Achromobacter UH*$_3$ takes up oxygen along a

FIG. 4.   The oxidation of γ-aminobutyrate by the cell-free extract from the γ-aminobutyric acid-adapted cells, in phosphate buffer solution and tris(hydroxymethyl)aminomethane-hydrochloride buffer solution.

In this case, the extraction of the cell-free enzyme was performed with distilled water, pH adjusted to 6.3. The other experimental conditions were the same as for Fig. 1, except that 2.0 ml. of the extract was used and the total volume was adjusted to 4.0 ml.

Curve A, phosphate buffer solution; B, tris(hydroxymethyl)aminomethane-hydrochloride buffer solution. The similar effects were observed also in the oxidation of glutamate.

sigmoid curve, if γ-aminobutyric acid is added as a substrate. The adaptation is remarkably inhibited in the presence of streptomycin and the inhibition is removed by preincubating the cells with the substrate. However, there has been no success in the extraction of an adaptive enzyme that attacks γ-aminobutyric acid itself. On the other hand, the oxidation of γ-aminobutyric acid by the cells is stimulated in rate by the additions of α-ketoglutaric acid or oxaloacetic acid, and the cells previously adapted to β-alanine can rapidly oxidize γ-aminobutyric acid with-

out any lag phase and streptomycin inhibition. It is difficult to consider that β-alanine might be oxidized via γ-aminobutyric acid. These facts suggest that adaptation to β-alanine causes adaptive increment of the intracellular enzymes probably of some members of TCA cycle-system, in their amount to supply the α-keto acids which are indispensable for transamination of γ-aminobutyric acid, and the increment results in that the cells adapted to β-alanine have already the ability to oxidize rapidly γ-aminobutyric acid without the lag phase and streptomycin inhibition. Really, this microörganism semiadaptively oxidize succinic, fumaric and malic acids, and these semiadaptations are strongly inhibited in the presence of streptomycin. Adaptive formation and degradation of succinic oxidase in the presence and absence of the substrate have been reported also in *Mycobacterium tuberculosis avium* (*10*), suggesting that the enzymes of TCA cycle in the living cells readily fluctuate in response to the alteration of surroundings. The cell-free extract from the cells not adapted to γ-aminobutyric acid takes up oxygen by the addition of γ-aminobutyric acid and α-ketoglutaric acid, but not by the former acid alone. However, the cell-free extract from the cells previously adapted to γ-aminobutyric acid consumes oxygen by the addition of γ-amino-butyric acid for a while, and the oxygen consumption increases in proportion to the amount of the extract added. It appears from these facts that the extract from the cells adapted to γ-aminobutyric acid contains some substance which can be oxidized only in the coexistence of γ-amino-butyric acid, and it is possible that the adaptation of the cells to γ-amino-butyric acid is an adaptive formation of the enzymes which produce such a substance. It is probable that this substance is α-ketoglutaric acid, since the oxidation of γ-aminobutyric acid by the living cells is remarkably stimulated in the presence of α-ketoglutaric acid. Based upon these experiments with living cells and cell-free extract, it seems that the adaptive oxidation of γ-aminobutyric acid by the living cells of *Achromobacter* results from adaptive increment of the enzymes, probably of some members of TCA cycle system, to supply additional α-ketoglutaric and oxaloacetic acid to the transaminase system already present in the cells adapted as well as non-adapted to γ-aminobutyric acid. An alternate assumption that an adaptive enzyme would be newly formed to attack γ-aminobutyric acid directly is less likely.

SUMMARY

The catabolic pathway of γ-aminobutyric acid has been studied with

Achromobacter UH₂ which adaptively oxidizes the acid.   In the living cells, the main catabolic pathway of γ-aminobutyric acid is its transaminations with α-ketoglutaric acid and oxaloacetic acid.   The relation between the adaptations to γ-aminobutyric acid and β-alanine was discussed in respect to adaptive formation of an enzyme system that produces the keto acids indispensable to the transamination of γ-aminobutyric acid.

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THE ROLE OF CALCIUM IN TAKA-AMYLASE A

By ATSUSHI OIKAWA and AKIO MAEDA

(*From the Laboratory of Biochemistry, Faculty of Science,
Osaka University, Osaka*)

The protective effect of calcium on some enzyme proteins has been reported by several authors (*1*). Yamamoto has pointed out that α-amylases belong to the enzyme of this type (*2*). Taka-amylase A*, an α-amylase produced by *Aspergillus oryzae*, was crystallised from aqueous acetone containing calcium acetate by Akabori *et al.* in 1954 (*3*). This crystal contains about ten atoms of calcium per molecule (*3, 4*). In its aqueous solution TAA is not inactivated by the addition of ethylenediaminetetraacetic acid* less than ten molar equivalents, but readily inactivated by an excess amount of EDTA (*5*). Recently, Tanaka obtained crystalline TAA-rivanol cymplex containing one mole of calcium and concluded that one molar equivalent of calcium was essential for the activity of TAA (*6*).

In the present work, the change in enzymatic activity and optical rotation of TAA solution accompanied by the removal of calcium has been studied and a possible role of calcium in TAA molecule has been discussed.

MATERIALS AND METHODS

TAA used in the present work was purified and recrystallised three or four times by Akabori's method (*3*).

Amylase activity was assayed by Fuwa's method (*7*) using amylose as substrate. The TAA sample was always diluted to the concentration of about 10 mg. per ml. and saccharifying power was determined at 0°, at which temperature the enzyme is entirely stable. Detailed procedure was as follows: 1.5 ml. of 0.2 per cent amylose solution was added into the mixture containing 1 ml. of adequately diluted TAA and 0.5 ml. of $M/2$ acetate buffer of the pH 5.3. After ten minutes at 0°*, the reaction was stopped

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* Hereafter, the following abbreviations are used; taka-amylase A, TAA; ethylenediaminetetraacetic acid, EDTA.

* This temperature was adopted in the principal experiment described in Table II. In other ones it was indicated in each description.

745

by 3 ml. of Somogyi's new reagent, and heated at 100° for 30 minutes. After cooling, 2 ml. of Nelson's reagent was added and the volume was filled up to 50 ml. The colour value of this solution was measured electrophotometrically. A unit of TAA activity (S. P.) was defined as that amount which increases reducing power equivalent to 0.1 mg. of glucose in ten minutes.

Optical rotation of TAA was measured at 0° and was indicated as $[\alpha]_D^0$.

The polarimeter tube was 20 cm. in length and mantled with a glass tube, in which ice-NaCl cooled water was circulated to maintain the temperature at 0°.

The concentration of TAA was determined by the micro-Kjeldahl method.

The Determination of the Calcium Content—An aliquot of TAA solution was dried and ashed in a platinum crucible. After adding 1 ml. of $N/10$ hydrochloric acid, it was dried again and dissolved in distilled water and assayed flame-photometrically.

Calculations are based on the assumption that the molecular weight of TAA is 53,000 (*3*).

RESULTS

During the dialysis of TAA solution in cellophane bag against $M/50$ sodium acetate at 5°, the molar ratio of calcium to TAA decreased rapidly from 10 to 1, while the enzymatic activity was not lowered, and even the slight increase was observed (Fig. 1). Under the condition that TAA contains more than 1 molar equivalent of calcium, TAA was stable at least for 22 hours at 37°. The amount of undialysable calcium was independent of the pH of the solution within the range, in which TAA is stable (Fig. 2). TAA solution containing 100 molar equivalents of EDTA was incubated at 5° for 48 hours, and then it was dialysed against $M/50$ sodium acetate, whereby TAA was inactivated almost completely. When the enzymatic activity decreased in the presence of an excess of EDTA the addition of 100 molar equivalents of calcium could not restore any lost activity (Table I).

Change of enzymatic activity and optical protatory power accompanied by the removal of calcium : 20 ml. of 3.34 per cent TAA solution was dialysed in cellophane bag against 800 ml. of $M/50$ sodium acetate, at 5°. The dialysate was exchanged with new solution of sodium acetate in every 24 hours. After 267 hours' dialysis, the temperature was lowered to 0° to prevent the inactivation which might be caused by the subsequent treatments. To this solution about 2 molar equivalents of EDTA was added and the dialysis was continued. During this dialysis the same amount of EDTA was added repeatedly at the times after 317, 365, 389, 412 and 461 hours from the begining of dialysis. The changes in enzymatic activity, optical rotation and calcium content during the
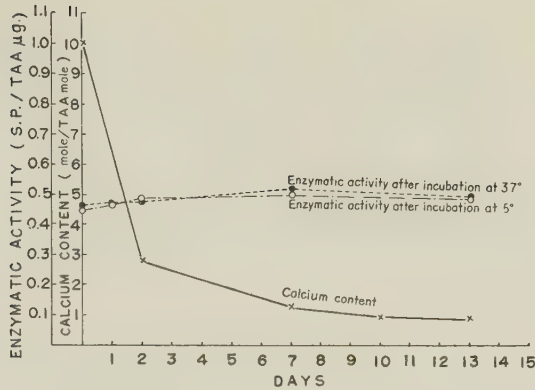
FIG. 1. Calcium content and specific activity of TAA during dialysis.

20 ml. of 3.22 per cent TAA solution was dialysed in cellophane bag against 500 ml. of Na-acetate solution (pH 7.0) at 5°. Dialysate was exchanged with new solution every day, after the sampling of dialysed solution for the measurement of Ca content and enzymatic activity. Enzymatic activity was measured at 1° after the dialysed solution was incubated with $M/10$ veronal buffer (pH 7.4) for 22 hours at 5° and 37°, respectively.

FIG. 2. Comparison of dialisability of calcium in various pH.
TAA was dialysed in cellophane bag against $M/50$ buffer solution at various pH indicated below.

        acetate buffer (pH 5.6) ●—●;
        veronal buffer (pH 6.8) . ○—○;
        veronal buffer (pH 8.2) ×—×.

above treatment were measured and the results are given in Table II.

Calcium content decreased rapidly to one mole, and was not lowered further until EDTA was added. The addition of EDTA to this solution, however,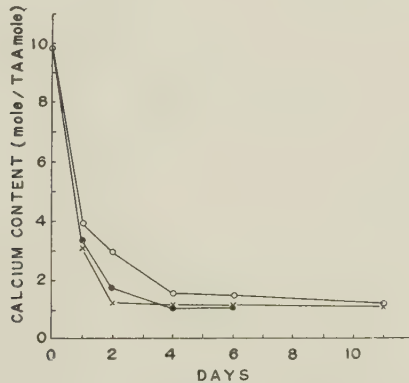 decreased this value again (Fig. 3). Enzymatic activity increased slightly during " dialysis without EDTA," and decreased gradually after the addition of EDTA. As for the optical rotation, the value was kept at its initial level during " dialysis without EDTA." However, after the addition of EDTA, levo-rotation increased rapidly. When the dialysis finished, TAA contained 0.21 mole of calcium and its activity was 30 per cent of maximal one.

TABLE   I

The Change of Calcium Content and Activity of Dialysed TAA after
the Addition of 100 Molar Equivalents of EDTA

	Dialysis with EDTA			Incubation with Ca
Time (*day*)	0	6	10	3
Specific activity ($S.P./\mu g$. at 37°)	2.92	0.34	0.09	0.12
Ca content (*mole/mole-TAA*)	—	0.16	0.06	

25 ml. of 1.74 per cent TAA solution was dialysed in cellophane bag against 3 liter of $M/50$ Na-acetate (pH 7.0) at 5°. Dialysed solution was incubated with 100 molar equivalents of EDTA at 5° for 48 hours. Dialysis was then carried out for 10 days. To the solution, thus obtained, 100 molar equivalents of Ca-acetate was added, and incubated for 3 days at 5°. Here, specific activity was shown as ($S.P./\mu g$.-TAA) at 37°.

On this dialysed solution some properties of TAA were examined. In 5 M urea its enzymatic activity was lost almost instantaneously, while undialysed TAA solution retains practically all of its activity under the same condition (Fig. 4). The stability of dialysed TAA was measured at 37° at various pH, and it was compared in the absence and in the presence of calcium (250 molar equivalents). In the presence of calcium there was no appreciable change in enzymatic activity, but in its absence the activity decreased remarkably (Fig. 5).

DISCUSSION

Among ten moles of calcium contained in crystalline TAA, one mole

TABLE II

Enzymatic Activity, Optical Rotation and Calcium Content of TAA During Dialysis

	day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Calcium Content	hours	0	27		77			141			214			283	316		354	389	410		458	480
	mole/mole	10.0	3.88		1.34			1.02			0.94			1.16	0.93		0.79	0.55	0.61		0.33	0.21
Specific Activity	hours	0	27		70			141			214				308	333	355	380	404	432		480
	$S.P./\mu g$	0.352	0.357		0.404			0.448			0.442				0.367	0.301	0.298	0.267	0.206	0.173		0.130
Optical Rotation	hours	0			72				169			242			308	332		380	404	428		480
	$[\alpha]_D$	-12.3			-12.0				-12.8			-12.2			-16.3	-18.7		-23.6	-25.6	-26.1		-25.4
Addition of EDTA	hours												267		317		365	389	412		461	
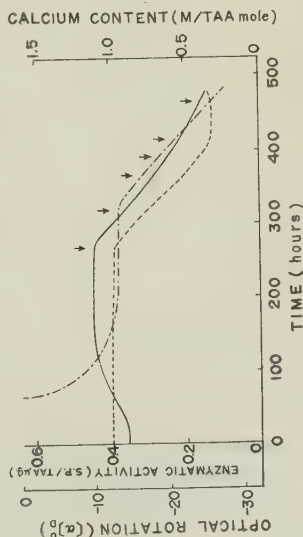
FIG. 3. Enzymatic activity, optical rotation and calcium content of TAA during dialysis.

20 ml. of 3.26 per cent TAA solution was dialysed in cellophane bag against 800 ml. of $M/50$ Na-acetate solution. Throughout all the procedure the temperature was kept below 8°, and at 0° after the addition of EDTA. In this experiment, redistilled water was used. ----- Ca content; - - - - optical rotation; ——— enzymatic activity. The arrows indicate the addition of EDTA (8 μM).

FIG. 4. Susceptibility of dialysed TAA to urea.

Dialysed and untreated TAA were incubated with 5 M urea at 37°, respectively. The enzymatic activity was assayed at 37° and showed as the percentage of initial activity. ——O—— dialysed TAA; --●-- untreated TAA; Here, the activity of untreated TAA showed tem porary increase. This phenomenon has been frequently observed.
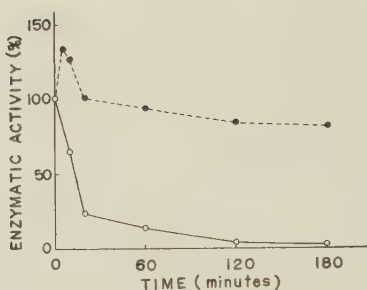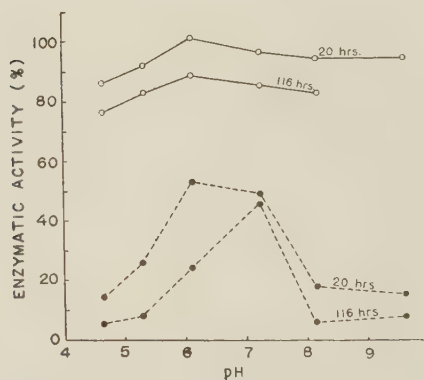

FIG. 5. Stability of dialysed TAA in the presence and in the absence of calcium.

Dialysed TAA was incubated in the various pH solution using Michaelis' veronal-acetate-HCl buffer in the presence and in the absence of about 250 molar equivalents of calcium at 37°. After the time indicated in the figure, enzymatic activity was assayed at 37°, and shown as the percentage of initial activity. O-O-O, contained 0.126 per cent of TAA, 6.25 mM of Ca-acetate and $M/10$ buffer solution. ●-●-●, had the same composition except the omission of calcium.

is non-dialysable in the usual dialysis. This last one mole of calcium was eliminated only by the dialysis in the presence of an excess of EDTA. The elimination of calcium except the last one mole does not cause any decrease of enzymatic activity, while by the further elimination of calcium with EDTA the enzyme is gradually inactivated, and levo-rotatory power of the solution is increased (Figs. 1 and 3). As the increase in levo-rotation is regarded as a signe of denaturation of proteins, the inactivation is considered to be not a direct but an indirect result of the elimination of calcium. That is, by the removal of the last one mole of calcium, TAA loses the key structure for stable configuration and then by unfolding of the peptide chain the optical rotation of TAA changes and the enzymatic activity is irreversibly decreased.

From the experimental results described above it is supposed that the calcium co-ordinates with a part of specific grouping of TAA protein (possibly with carboxyl group, and hydroxy group of serine and threonine or phenol residue of tyrosine), resulting in the stabilisation of structure of TAA molecule.

To elucidate the role of the calcium in TAA, *i.e.*, whether it is structural only or both structural and cofactor-like, further investigation is required.

SUMMARY

1.   Calcium salt of taka-amylase A contains ten moles of calcium. One mole of them remained after dialysis against $M/50$ sodium acetate solution. Its enzymatic activity and optical rotation did not change significantly during the dialysis.

2.   The last one mole of calcium could be removed by the dialysis in the presence of EDTA. The elimination of this calcium was accompanied by the increase of levo-rotation of TAA and the loss of enzymatic activity.

3.   TAA dialysed in the presence of EDTA was less stable against heat and urea than calcium-containing one.

4.   From these experimental results it was concluded that one mole of calcium is essential for the maintenance of active structure of TAA.

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STUDIES ON RIBONUCLEASES IN TAKADIASTASE. I

By KIMIKO SATO and FUJIO EGAMI

(*From the Laboratory of Biochemistry, Faculty of Science,
University of Nagoya, Nagoya*)

Since W a l t e r J o n e s discovered a thermostable enzyme in the pancreas which attacks ribonucleic acid (RNA) (*1*), tissue homogenates and soluble enzymes from various sources have been found to hydrolyse RNA.   They may be classified as nonspecific phosphodiesterases found in the spleen, snake venom, *etc.*, and specific ribonucleases found in the pancreas, spleen (*2*), pea leaves (*3*), *etc.*   The best characterized of them is crystalline pancreatic ribonuclease (RNase I) (*4–7*).   The studies on the specificity of RNase I and the others have largely contributed to the elucidation of the structure of RNA (*8*).

Recently in Japan RNase of *Aspergillus oryzae* has been described by K u n i n a k a (*9*) and S a r u n o (*10*) independently.   They studied various enzymatic characteristics of the RNase.   Although S a r u n o reported that specific RNase was obtained in the crystalline state from Takadiastase, it was shown to be far from homogeneous in electrophoresis.   So the present investigation was undertaken in order to purify the RNase of Takadiastase and to study its enzymatic characteristics.   In the present paper the existence of two ribonucleases in Takadiastase, their partial purification, a preliminary study of their specificity and other enzymatic characteristics will be described.

METHODS

Measurement of Enzyme Activities

Ribonuclease—0.5 ml. of enzyme solution in 0.2 M Tris buffer (pH=7.5) or 0.2 M acetate buffer (pH=4.5) which contains 4×10^{-3} M EDTA was mixed with 0.5 ml. of RNA (Schwarz, dialyzed and lyophilized, and contains 8.6 per cent phosphorus) solution (6 mg./ml.).   After incubation for 15 minutes at 30° the reaction was stopped with 0.2 ml. of 0.75 per cent uranyl acetate in 25 per cent perchloric acid.   After standing for 30 minutes the precipitate was removed by centrifuging.   0.25 ml. of the supernatant fluid was diluted to 5.0 ml. with water and the optical density was read at 260 mμ in the Beckman spectrophotometer against the blank without enzyme

(*11*).   It was desirable to restrict the amount of enzyme so that the net optical density
of the final diluted sample did not exceed 0.300 (6 units).   In some cases, to 2.0 ml.
of the same reaction mixture the same volume of 0.25 per cent uranyl acetate in 2.5
per cent trichloroacetic acid was added and kept for 30 minutes.   After the removal
of the precipitate, 2.0 ml. of the supernatant fluid was taken out and organic phos-
phorus was measured by the method of Allen (*12*).

   Definition of RNase Unit—One unit of enzyme activity was defined to be the amount
of RNase which produced 10^{-4} mg. of P. for 1 minute in 1 ml. of reaction mixture under
the conditions described above.   The amount of P. was known by direct measurement
or by using the standard curve which showed the relation between the amount of
P. and optical density at 260 mμ (Fig. 1).   The specific activity was defined as units
per mg. protein nitrogen (*13*).
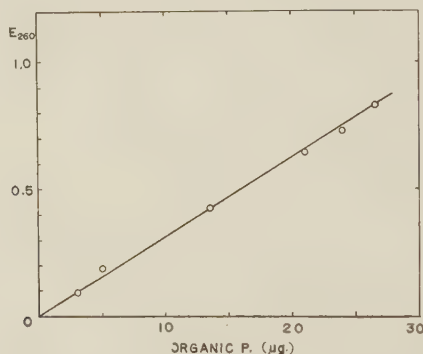
FIG. 1.   Relation of optical density at 260 mμ to organic P.
in uranium reagent soluble fraction.   Reaction was stopped by uranium
perchloric acid reagent and optical density at 260 mμ was measured,
while it was stopped by uranium trichloracetic acid reagent and
organic P. was measured.   For details of procedure, see the text.

   Deoxyribonuclease—It was determined by the method of Kunitz (*14*).

   *Phosphodiesterase*X1 ml. of enzyme solution was mixed with 1 ml. of diphenyl phos-
phate solution (1 mg./ml.) and incubated for 2 hours at 30°.   After removing the pro-
tein by trichloroacetic acid and neutralizing with NaOH, the phenol produced was
measured by the method of Lowly *et al.* (*15*).

   Nucleotidase—A solution of mononucleotides mixture was incubated with the
enzyme solution at 30° and the inorganic P. produced was measured.

Paper Chromatography of Hydrolysate

   For the identification of mononucleotides and 2′, 3′-isomers of purine nucleotides,
the developing solvent used was saturated ammonium sulfate : water : isopropanol
(79 : 19 : 2) (Solvent 1) (*16*).   In this chromatography pyrimidine mononucleotides

give one spot, therefore in order to find the ratio of cytidylic acid and uridylic acid, the spot was extracted, and the ratio was calculated from the optical density at 260 mμ and 278 mμ. For the purpose of finding the degree of hydrolysis, Solvent 2 (*17*) was used, consisting of isopropanol : water (7 : 3). The paper used was Tôyôroshi No. 51. After drying the paper was put upon a photographic printing paper (Mitsubishi CH) in a dark room and exposed to light (Mazda sterillizing lamp 15 w.) through U. V. filter (257.3 mμ) of Scientific Research Institute.[1] By this treatment a chromatogram which revealed white spots against a black background was obtained.

Zone Electrophoresis

Zone electrophoresis was carried out with potato starch as the supporting material in a trench $40 \times 2 \times 1$ cm. After dialysis overnight against distilled water in the cold room, 0.6 ml. of the enzyme preparation containing about 10,000–50,000 units was put on the band of 1 cm. wide of the starch column, after running for several hours each band of 1 cm. wide was eluted with 2 ml. of distilled water. 0.1 ml. of supernatant fluid was used for measurement of activity.

EXPERIMENTAL

Presence of Two RNases in Takadiastase

Crude extract of " Takadiastase Sankyo " was subjected to zone electrophoresis in veronal buffer at pH 8.5 for 5 hours at 300 volts. The pattern obtained is presented in Fig. 2 (a). Zone electrophoretic pattern with the preparation partially purified by heat treatment and ammonium sulfate fractionation is shown in Fig. 2 (b). After these treatment this enzyme preparation could not hydrolyse DNA, diphenyl phosphate and mononucleotides. From these electrophoretic patterns it seems that in Takadiastase there exist at least three enzymes which can hydrolyse RNA, and two of them may be regarded as specific RNases. We propose to call them RNase T_1 (major component) and RNase T_2 (minor component), respectively. RNase T_1 and RNase T_2 are both thermostable, but T_1 has its pH optimum at pH 7.5, while T_2 has it near 4.5. Studies on the third component of these enzymes have not yet been carried out.

Purification of RNase T_1

Purification of RNase T_1 was performed by the following procedure

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1) Kindly supplied from the laboratory of Dr. E. I w a s e of Scientific Research Institute.
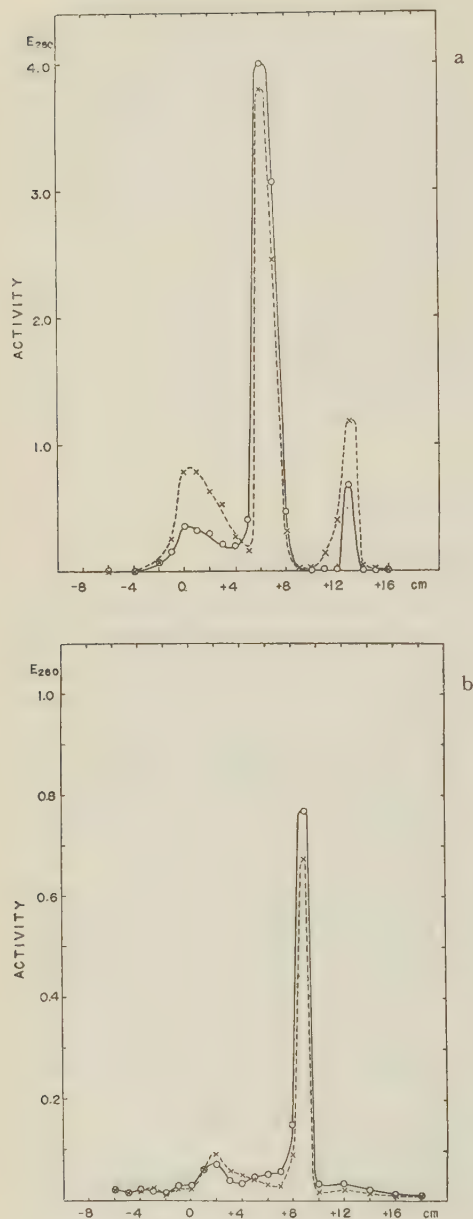
Fig. 2. Zone electrophoretic patterns of RNase activity of Taka-diastase on starch column (total units used were about 80,000) ; in veronal buffer, pH 8.5, $\mu=0.1$; at a field strength of 300 volts/40 cm. (a) Crude extract ; after 5 hours electrophoresis. (b) Preparation partialy purified by heating and ammonium sulfate fractionation ; after 6 hours electrophoresis. The solid lines represent RNase activity measured by at pH 7.5 and broken lines at pH 4.5.

(Scheme 1).

Heat Treatment—The enzyme in purified state is stable against heat treatment in the medium adjacent to pH 6 as is shown later, however in the crude extract of Takadiastase it is more stable at pH 2.5 as shown in Fig. 3.   Therfore at first 100 g. of " Takadiastase Sankyo " was ex-

Scheme   1

Purification of RNase T_2

100 g. " Takadiastase Sankyo " taken up in 500 ml. of dist. water
$+$HCl to pH 2.5 heated to 80°, 2 min.
$(NH_4)_2SO_4$ ppt.   after adjusting to pH 6

ppt. at 45% sat.
(discarded)

filtrate$+(NH_4)_2SO_4$ to sat.

ppt. taken up in 0.1 M citrate (pH 6)

filtrate
(discarded)

refractionation with $(NH_4)_2SO_4$

ppt. at 50% sat.
(discarded)

ppt. at sat. taken up in citrate

dialysis

adsorption with calcium phosphate gel
in 0.01 M acetate (pH 5.6)

gel

fluid*

eluted with 0.05 M Na_2HPO_4

gel
(discarded)

fluid

dialysis

$+$active charcoal in
0.02 M acetate (pH 5.6)

charcoal
(discarded)

filtrate
purified RNase T_1

* When the specific activity before this treatment was less than 40-fold, most part of RNase T_1 was obtained from this fluid, and purified to about 50-fold.   Therefore this fluid was again subjected to gel treatment.

tracted with 500 ml. of distilled water.   The mixture was adjusted to pH 2.5 with 6 N HCl (about 50 ml.) and was heated in a boiling water bath until the temperature of the solution reached 80°.

It was kept at 80° for two minutes.   It was then quickly cooled in an ice-water bath.   After cooling the mixture was adjusted to pH 6 with

(1:1) ammonium hydroxide.   Unless this was heated, the activity of
RNase was often lost during the dialysis which followed.   This pro-
cedure was also advantageous to inactivate coexisting DNase, nucleoti-
dase, protease, cellulase, *etc.*

Ammonium Sulfate Fractionation—After heating the extract, 170 g. of
solid ammonium sulfate was added, and precipitate was removed after
one hour by filtration with the aid of a large quantity of Hyflo Super Cel.
The supernatant solution was brought to saturation by the addition of
more ammonium sulfate, and after 3 hours the precipitate was collected
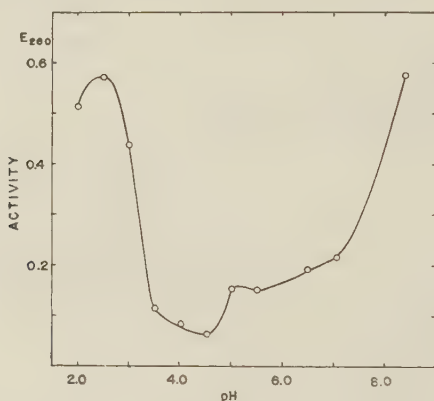by filtration on Hyflo Super Cel.   The precipitate was dissolved in pH 6

FIG. 3.   Effect of pH on the thermostability of crude extract of
Takadiastase. The extract adjusted at each pH was heated to 80°
for 5 minutes, after cooling and removal of precipitate by centrifuging,
aliquot of the supernatant was taken and RNase activity was measured.

citrate buffer.   To this clear reddish brown solution, ammonium sulfate
(380 g. per liter) was again added, and after filtration the supernatant
solution was brought to saturation.   The precipitate produced was col-
lected, dissolved in the buffer and dialysed against distilled water in the
cold room.   (Following dialysis were carried out in the same way.)

Adsorption with Calcium Phosphate Gel—After dialysis (ammonium ion
free) the solution was adjusted to pH 5.6 with acetate buffer (final 0.02
M).   The protein concentration was about 2–3 mg. per 1 ml., and the
specific activity of RNase was above 50-fold that of the crude extract.
An equal volume of calcium phosphate gel suspension (prepared by the

method of S u m n e r (*18*), 25–40 mg. dry weight per 1 ml.) was added and kept for 10–15 minutes. At once the adsorption complex was centrifuged down and the supernatant was discarded. Elution of the enzyme was performed with as small a quantity as possible of 0.05 M sodium hydrogen phosphate, and elution was repeated twice more. The eluates were combined and dialysed. Before the gel treatment was carried out, if the specific activity of the preparation did not exceed 40-fold, an appropriate amount of the gel was added to adsorb inactive proteins and the adsorption complex was removed by centrifuging. Then the ordinary gel treatment described above was carried out. After this step of purification, the specific activity of the preparation was about 190–200 fold. By this treatment RNase T_1 was separated from RNase T_2.

TABLE I

Purification of RNase T_1

	Over-all yield*	Specific activity*
Crude extract	100%	650
Heat treatment	—	—
Ammonium sulfate	25	40,100
Gel treatment	14	145,000
Active charcoal	12	240,000

* Over-all yield and specific activity of RNase T_1 were calculated by using the ratio of the activity depending on RNase T_1 to whole RNase activity found from the patterns of zone electrophoresis.

Treatment with Active Charcoal—After dialysis the enzyme solution was adjusted to pH 5.6 with acetate buffer (final 0.02 M) and an appropriate amount of active charcoal (Norit Extra) was added, and filtered. When too much Norit was added, RNase T_1 was adsorbed.

The filtrate was dialysed against distilled water and concentrated carefully *in vacuo*. The specific activity of this preparation was 240,000 and 370-fold with a yield of 12 per cent, and about 12 mg. of enzyme protein was obtained from 100 g. of Takadiastase. The procedure was found to be quite reproducible. The results of a typical fractionation experiment are given in Table I. The specific activity of RNase I (comercial crystalline enzyme from Worthington Biochemical Sales Co.) was determined under the same conditions and found to be 700,000.

Homogeneity of RNase T_1—The homogeneity of the most purified preparation of RNase T_1 (specific activity 240,000 at pH optimum) was tested by zone electrophoresis in veronal buffer at pH 8.5, $\mu=0.1$, and in acetate buffer at pH 5.6, $\mu=0.1$, at 240 volts for 5, 6.5 hours, respectively. The result is shown in Fig. 4. The peak of RNase activity was single and agreed with the peak of protein. The isoelectric point of this RNase T_1 seems to be in the acid pH region. This preparation
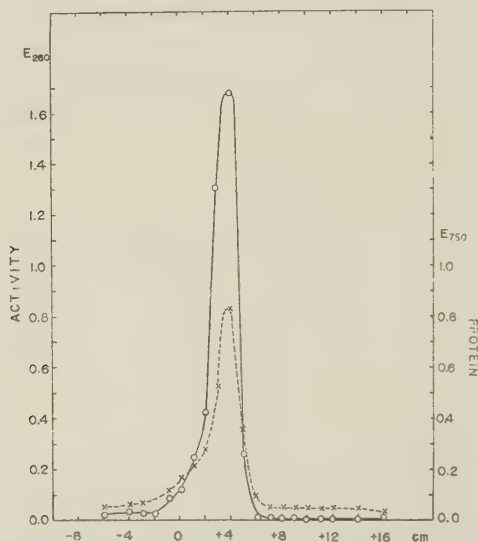

FIG. 4. Zone electrophoretic pattern on the starch column obtained with the most purified RNase T_1 ; in veronal buffer, $\mu=0.1$, pH 8.5 ; at a field strength of 240 volts/40 cm. ; 5 hours electrophresis. 0.1 ml. of eluates from each band was used for determination of activity (solid line), and 1.0 ml. of it was used for measuring protein (broken line).

seems to be free from RNase T_2, because the peak corresponding to RNase T_2 was not found and it has no activity hydrolysing adenosine-2′, 3′-phosphate (cyclic adenylic acid) to 3′-adenylic acid.

By boundary electrophoresis this preparation is shown to contain very small amount of impurity. This impurity seems to be of a non protein nature, because it could not be detected by the above described zone electrophoresis. The sedimentation measurement on RNase T_1

was made in the analytical ultracentrifuge (Spinco, model E).[2] A single, slowly sedimenting boundary was observed, but the asymmetry of this peak showed that a small quantity of a lighter component exists in it. These results show that RNase T_1 is in an almost homogeneous state and free from RNase T_2 and other enzymes so far tested. However further purification must be carried out.

Properties of RNase T_1

Stability—The most purified preparation, when heated to 80° for 5 minutes at various pH, was found to be most stable at pH 6. At the

FIG. 5. Effect of pH on rate of hydrolysis of RNA by RNase T_1. The final concentration of buffer was 0.05 M. The symbols are ×, acetate buffer ; ○, phosphate buffer ; △, Tris buffer.
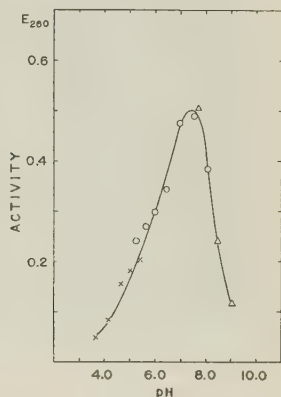
most stable pH the activity was not lost at all after heating to 100° for 10 minutes (protein concentration was about 5 μg. per 1 ml. 0.01 M of pH 6 citrate buffer). This preparation was tested in storage under various pH for a week in a cold room (protein concentration was about 10 μg. per 1 ml.). It was also most stable at pH 6, and under this condition no loss of activity was observed.

Effect of pH—The influence of pH on enzyme activity in three different buffers is shown in Fig. 5. The optimal pH is 7.5.

Inhibitors and Activators—This RNase was inhibited by various metal ions and activated by EDTA. Therefore, at the measurement of activity, EDTA was added to the reaction mixture. It is interesting that the same concentration of Mg ion, which activates RNase I and splenic

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2) Measured by Dr. N. U i, to whom our thanks are due.

RNase (*19*, *2*), inhibits RNase T_1. Monoiodoacetate which inhibits RNase I (*20*), does not inhibit RNase T_1 (Table II).

Specificity—To find out the specificity of RNase T_1, using the most purified enzyme, digestion of RNA was pursued by measuring the organic P. and paper chromatography.

In the first stage of digestion, uranium reagent soluble organic P. increased rapidly, but later it increased very slowly. When the digestion was performed for 48 hours using 1000 times of enzyme used in the ordinary measurement of activity, 30 per cent of uranium reagent

TABLE II

Activation and Inhibition by Some Reagents

R t	Final concentration (-log M)	Activation (%)
$MgCl_2$	5	−15
	3	−25
	1	−40
$CaCl_2$	2	−30
NaCl	1	0
$FeCl_3$	3	−40
$ZnSO_4$	3	−15
$CuSO_4$	3	−50
NaF	2	0
ICH_2COONa	3	0
EDTA	3	+25
NoN_3	3	0

Activity was measured without EDTA, pH 7.5, using the enzyme in most purified state (4 units).

insoluble fraction remained in the reaction mixture. The enzyme could hydrolyse the 'core' of RNase I, and even faster in the early stage of hydrolysis than RNA. So RNase T_1 must be different from RNase I in its specificity.

To test the reaction products, aliquots were removed at intervals and chromatographed in Solvent 1. As shown in Fig. 6, (a), spot 2' (middle of 2 and 3) and 4 appeared at first (see II, VI). The spot 2' increased gradually, later decreased, and finaly vanished with the simultaneous appearance of spot 4, guanylic acid. It revealed fluorescence with

vapour of HCl. Therefore it must be a derivative of guanylic acid. The small amount of it exists in the RNase I core, see V. Among the spots of mononucleotides the spot of 3′-guanylic acid (spot 4) had already
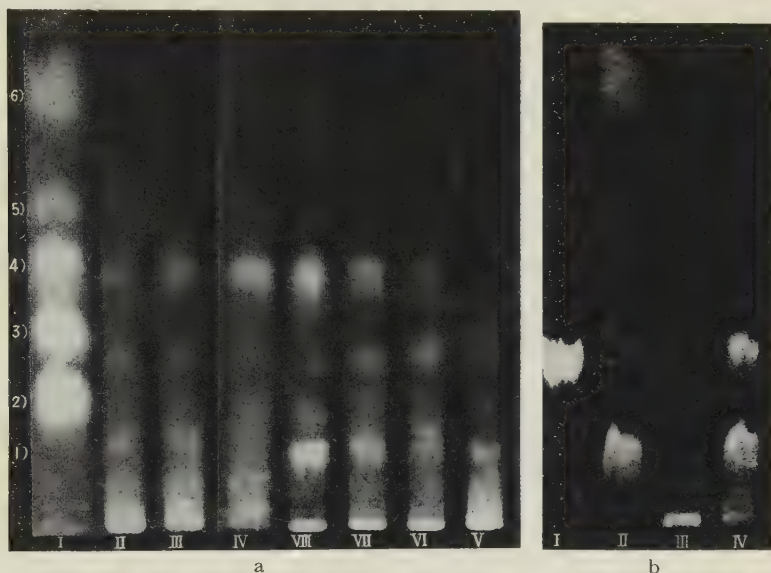

Fig. 6. Paper chromatograms of the digestion products by purified RNase T₁.

(a) Time course of the digestion of RNA and RNase I core. I : authentic samples of mononucleotides. The spots are; (1), certain dinucleotides and cyclic adenylic acid ; (2), 3′-adenylic acid ; (3), 2′-adenylic acid ; (4), 3′-guanylic acid ; (5), 2′-guanylic acid ; (6), pyrimidine nucleotides. II : after 30 minutes, III : after 4 hours, IV : after 24 hours digestion of RNA. V : time zero, VI : after 30 minutes, VII : after 4 hours, VIII : after 24 hours digestion of RNase I core. The digestion was carried out in Tris buffer pH 7.5 at 30°. The amount of enzyme was 340 units. Solvent 1 was used.

(b) Hydrolysis of synthetic RNA (II), polyadenylic acid (III) and natural RNA (IV) by purified RNase T₁ for 24 hours. I : known adenylic acid. Solvent 2 was used. Enzyme used was 340 units.

appeared after 30 minutes, while other mononucleotides were scarcely liberated even after 24 hours of digestion (see IV, VIII). When the core of RNase I was used as substrate (V, VI, VII, VIII), after one day

spot 1 was clearly revealed, R_f value of the spot corresponds to that of adenosine-2′, 3′-phosphate, and certain dinucleotides. When the amount of enzyme was increased (10-fold of the experiment shown above), and digestion was carried out for 48 hours at 30°, adenylic acid appeared and a small amount of pyrimidine nucleotides, too. Among the pyrimidine nucleotides which appeared very slowly, cytidylic acid appeared more slowly than uridylic acid. The molar ratio of cytidylic acid to uridylic acid was 0.5 after 24 hours digestion and 1.0 after 48 hours.

Synthetic polyadenylic acid and polynucleotide AGUC prepared by polynucleotide phosphorylase from the equal amount of four nucleoside diphosphates, (22, 23) were subjected to the action of RNase T_1. The former was scarcely digested and a small amount of adenylic acid was liberated after 24 hours. In contrast the latter was digested as easily as natural yeast RNA. It may be concluded from these results that RNase T_1 hydrolyses secondary phosphate ester of guanosine-3′-phosphate much faster than esters of other nucleotides, and produces 3′-nucleotides. It has not yet been established whether RNase T_1 produces nucleoside-2′, 3′-phosphate during the digestion of RNA or not. At any rate such cyclic esters have not yet been obtained from the digestion mixture, and RNase T_1 could hardly hydrolyse cyclic adenylic acid and cyclic cytidylic acid (21) in 24 hours. Cyclic guanylic acid and cyclic uridylic acid have to be tested in order to clarify the specificity on this point.

Difference between RNase T_1 and RNase T_2

It seems that RNase T_2 has pH optimum near 4.5 and its isoelectric point is near pH 7. The enzyme preparation after ammonium sulfate fractionation was subjected to zone electrophoresis as described above, and the fraction comprising the peaks of RNase T_1 and T_2 were eluted separately, and these solutions were used as enzyme preparations. With these enzyme preparations cyclic adenylic acid was incubated, at 30°, in pH 7.5 and pH 4.5, and after 3 hours aliquots of the reaction mixture were chromatographed in Solvent 1. The result obtained by this experiment is shown in Fig. 7. When RNA was digested by RNase T_2, contrary to RNase T_1, in the early stage of digestion adenylic acid and pyrimidine mononucleotides appeared in the reaction mixture, but guanylic acid appeared slowly. Mononucleotides produced were also 3′-phosphates. RNase T_2 preparation was again heated to 80° for 5 minutes but the enzyme activity did not change at all. RNase T_1 mixed with a small amount of RNase T_2 was incubated with RNA at 30°, then

RNA was quickly and completely digested to four mononucleotides.

It was concluded by these experiments that RNase T_1 and RNase T_2 are both thermostable specific RNases, but are different in their physical properties and specificities.
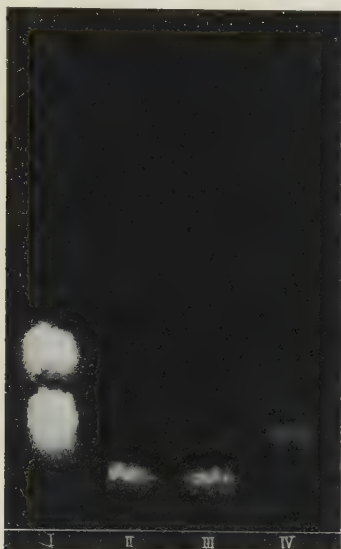

Fig. 7. Paper chromatogram showing the difference of RNase T_1 and RNase T_2 in their specificities against cyclic adenylic acid. As the enzyme preparations the eluates from each peak of zone electrophoretic pattern were used.

I : authentic sample of adenylic acid (mixture of 2′, 3′-isomers II : synthetic cyclic adenylic acid. III : cyclic adenylic acid incubated with RNase T_1 for 3 hours at 30° in Tris buffer (pH 7.5) or acetate buffer (pH 4.5). IV : cyclic adenylic acid incubated with RNase T_2 for 3 hours at 30°. Solvent 1 was used. Enzyme units used were both 50 units.

DISCUSSION

S a r u n o reported that his crystalline *Aspergillus* RNase has pH optimum near 4.5 (*10*), and its isoelectric point is near pH 7[3]. Considering these facts with the results obtained by the above experiments,

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3) Personal Communication.

it seems that RNase in S a r u n o's preparation corresponds to RNase T_2. There is no evidence that his enzyme is not contaminated with RNase T_1, therefore the specificity of pure RNase T_2 should be investigated later.

It is very interesting that in the same source there are two different thermostable RNases. These two RNases differ in their specificities from RNase I, leaf RNase, spleen phosphodiesterase, and snake venom phosphodiesterase, *etc*. In fact RNase T_1 quickly produces guanylic acid from RNA and RNase I core, but very slowly produces adenylic acid and pyrimidine nucleotides, and hydrolyses with difficulty, if any, cyclic cytidylic acid and cyclic adenylic acid. RNase T_2 in contrast to RNase T_1 quickly produces adenylic acid and pyrimidine nucleotides, and can easily hydrolyse cyclic adenylic acid producing 3'-adenylic acid. It is necessary, therefore, to obtain perfectly pure RNase T_1 and T_2 or at least to separate from each other, and determine their specificities in detail. Then, they may be used for the study of the structure of polynucleotides.

The mixture of RNase T_1 and RNase T_2 preparation can digest RNA very quickly to 3'-mononucleotides, so they may be used for the elimination of RNA contaminating in protein, *etc*.

The molecular weight of RNase T_1 was estimated to be about 10,000 or more from the measurement of its osmotic pressure[4] and its sedimentation measurement by analytical ultracentrifuge (Spinco, model E).[2] So RNase T_1 seems to be a favourable material for the study of the chemical structure of enzyme protein and the relation between chemical structure and enzyme activity just as done in the case of RNase I.

SUMMARY

1. There exist two different RNases in Takadiastase as was shown by zone electrophoresis on starch column. These two enzymes are both thermostable. They were named RNase T_1 (major component) and RNase T_2 (minor component).

2. RNase T_1 has been purified 370-fold, by the following procedure ; heat treatment, ammonium sulfate fractionation, adsorption with calcium phosphate gel and treatment with active charcoal. This enzyme was obtained in almost homogeneous state and free from RNase T_2.

3. The optimal pH of RNase T_1 is 7.5, and it is stable near pH 6

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4)   Measured by D. K. H o t t a, to whom our thanks are due.

for heating and long storage.

4. The specificity of RNase T_1 is different from that of RNase I. RNase T_1, digesting RNA or RNase I core, produces 3'-guanylic acid much faster than other mononucleotides.

5. RNase T_1 is inhibited by various metal ions, *e.g.*, Mg, Ca, Fe, Zn, Cu, *etc.*, and activated by EDTA. Monoiodoacetate has no effect on RNase T_1.

6. RNase T_2 has its pH optimum near pH 4.5, and is also thermostable. It is different from RNase T_1 and RNase I in its specificity, *e.g.*, it liberates adenylic acid and pyrimidine mononucleotides quickly from RNA.

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PHYSIOLOGICAL CHEMISTRY OF THE HARD TISSUES

III. METABOLISM AND DISTRIBUTION OF GLYCEROPHOS-PHATE-P^{32} AND ITS INCORPORATION INTO RAT INCISOR

By YOSOJI ITO, SUSUMU TSURUFUJI and YOSIO HIRAMATSU

(From the Department of Physiological Chemistry, Departments of Pharmaceutical Sciences, Faculty of Medicine, University of Tokyo, Tokyo)

Since R o b i s o n proposed a theory on the mechanism of calcification (*1*), *i.e.*, local concentration of free inorganic phosphate ion increases to be precipitated as a bone salt by hydrolysis of organic phosphate esters by the activity of alkaline phosphatases in actively calcifying regions, numerous works have been reported on this question. In spite of laborious investigations, these reports are confusing and the role of phosphate esters and alkaline phosphatase on the mechanism of calcification is still obscure. *In vitro* calcification techniques have been extensively used to examine the effect of various factors on the activity of calcification, but calcifiable cartilage has been the only materials used for the examinations, and essentially calcifiable tissues, *i.e.*, osteoblastic, odontoblastic, and ameloblastic tissues have not been used, either *in vitro* or *in vivo* studies, because of technical difficulties.

Using glycerophosphate -P^{32} as a tool, the present study was carried out to investigate contribution of ester phosphate to the process of calcification in *in vivo* experiment. In agreement with the previous study (*2*) continuously growing incisor of rat was indicated to be an excellent material for the present purposes.

EXPERIMENTAL

Synthesis and Identification of Glycerophosphate-P^{32}—A mixture of α- and β-glycerophosphate labeled with P^{32} was prepared synthetically through a procedure based on the method described by S p i n k s *et al.* (*3*). About 10 mc. of carrier-free inorganic phosphate-P^{32} solution in a 15 ml. centrifuging tube was added with 15 mg. of NaH$_2$-PO$_4$·2H$_2$O. The solution was evaporated to dryness and heated overnight at 110 to 120° for complete dehydration. About 0.1 g. of desiccated glycerol was added to the dried mass, and the mixture was heated at 175 to 190° for 2 hours in vacuum. A resultant viscous mass, (C$_3$H$_7$O$_2$)$_2$·PO$_4$Na, was hydrolysed into C$_3$H$_7$O$_2$·PO$_4$Na$_2$ by

refluxing with a calculated amount of 10 per cent NaOH. Free inorganic phosphate-P^{32} in the reaction mixture was precipitated in the centrifuging tube used as a reaction vessel with a calculated amount of $CaCl_2$ and eliminated by centrifugation. Trisodium phosphate was added to the supernatant as a scavenger for remaining inorganic phosphate-P^{32} and then the scavenger was precipitated by adding calculated amount of $CaCl_2$, repeating this procedure three times in oder to eliminate completely any trace of inorganic phosphate-P^{32}. Identification of glycerophosphate-P^{32} was performed with unidimensional paper chromatography by using autoradiographic examination according to the procedure described by S e k i g u c h i (4). B a n d u r s k i and A x e l - rod's procedure (5) for developing the color of phosphorus compounds on paper chromatogram was used to refer the autoradiogram to the paper chromatogram of non-labeled inorganic phosphate and α- and β-glycerophosphate.

No contamination with inorganic radiophosphate was detected in the auto-radiogram. Main spots consisted of α- and β-glycerophosphate. Two weak spots of unidentified impurity showing lower R_f values than the main spots were found. Radioactivity assay with a G-M counter indicated that the activity of the impurity corresponded, respectively, to 3 and 0.4 per cent of the combined activity of the main spots. Therefore, radioactive purity of the labeled glycerophosphate is sufficient for the present biochemical studies. The specific activity of the preparation was so high that no interference to phosphorus metabolism in experimental animals would have occured.

Radioactivity yield of glycerophosphate was about 50 per cent of the cal-culated value.

Determination of Labeled Phosphorus Compounds—Urine samples were directly collected with ureteral cannula in rabbits. All samples of blood and urine were chilled im-mediately after collecting them to check any possible changes in the distribution and chemical form of P^{32}-compounds. Two ml. of urine or serum was added to 4 ml. of 12 w/v per cent trichloroacetic acid (TCA) solution. Each 1 ml. of the TCA super-natant was used for the radioactivity assay for acid-soluble organic and inorganic phosphorus and the determination of inorganic phosphate. Duplicated assays were performed in all determinations. For the determination of inorganic phosphate T a k a h a s h i's butanol-ascorbic acid method (6) was used. Complete separation of inorganic phosphate-P^{32} and acid-soluble organic phosphate-P^{32} (glycerophosphate-P^{32}) in the TCA supernatant for radioassay was achieved by precipitating inorganic radio-phosphate as ammonium magnesium phosphate after adding non-active inorganic phosphate and glycerophosphate as a carrier. Reprecipitation of labeled inorganic phosphate was performed in the presence of non-active glycerophosphate. All radio-activity assays were corrected for radioactive decay, geometrical efficiency, back scattering, absorption of β-particle and for the variation of efficiency of G-M counter.

RESULTS AND DISCUSSION

Disappearance and Hydrolysis of Labeled Glycerophosphate in Blood Stream

—Three male adult rabbits weighing about 2.5 kg. were given in-
travenously 25 to 40 μc. of glycerophosphate-P^{32} containing 37 μg. of
carrier as phosphorus in 0.6 per cent sodium chloride solution.   Blood
samples were drawn 20, 60, 120, and 180 minuts after the injection and
urines were collected simultaneously.

Results of radioactivity assay of inorganic and acid-soluble organic
phosphorus fractions of the serum are summarized in Table I.   Twenty
minutes after the intravenous injection of glycerophosphate, radioactivity
of inorganic phosphate in the serum exceeded that of acid-soluble organic
phosphate fraction (glycerophosphate-P^{32}).   With passage of time radio-

TABLE   I

Organic and Inorganic Radiophosphates in Trichloroacetic Acid Supernatant
of Rabbits Sera

Each rabbit was given glycerophosphate-P^{32} with high specific
activity with intravenous injection.

Time after injection	Fractions	Counts per minute in aliquot of fraction		
		Rabbit I	Rabbit II	Rabbit III
mins. 20	Inorganic	667	522	427
	Organic	557	450	352
60	Inorganic	351	195	165
	Organic	253	165	156
120	Inorganic	106	62	75
	Organic	47	31	68
180	Inorganic	85	68	—
	Organic	11	17	—

activity of both fractions fell rapidly.   Thus very rapid hydrolysis of
glycerophosphate in the serum may be considered.

Table II gives specific activity values of inorganic phosphorus of
the sera and those of corresponding urines.   The values in urines at 20
and 60 minutes were higher than those of the sera.   This observation
indicates rapid hydrolysis of glycerophosphate-P^{32} in the kidney.

Detailed examination was attempted to confirm the metabolism of
glycerophosphate in the blood serum.   Forty-six male, growing rats
were divided into two groups.   The rats in the one group were given
intravenously 1 to 5 μc. of glycerophosphate-P^{32} which contained 0.6

to 6 μg. of carrier as phosphorus.   Rats in the other group were given
similarly inorganic phosphate-P[32].   Each three to twelve rats from both
groups were sacrificed 5, 8.5, 20, and 40 minutes after the injection by
cutting the carotid artery and the blood was collected from each rat.
At the same time 0.5 ml. of blood was also drawn 3 minutes after the
injection from the femoral artery in the group sacrificed at 8.5 minutes.

    The radioactivity measurements for the serum phophorus are shown
in Fig. 1.   Based on the data on the total volume of serum of a rat by
Berlin *et al.* (7), percentage recovery of radiophophorus as inorganic
and acid-soluble organic phosphates remaining in total serum is calcu-
lated from the original measurements and plotted against time.

TABLE   II

Specific Activity of Inorganic Phosphorus in Serum and Urine of Rabbits

Each rabbits was given glycerophosphate-P[32] with intravenous
injection.   Urine was collected with ureteral cannula.

Time after injection	Sources	Specific activity of inorganic phosphorus		
		Rabbit I	Rabbit II	Rabbit III
mins.		(c.p.m. $\times 10^{-2}$ per mg. of P)		
20	Serum	267	223	158
	Urine	583	796	—
60	Serum	141	83	66
	Urine	154	132	273
120	Serum	42	26	34
	Urine	30	46	41
180	Serum	34	29	—
	Urine	24	24	—

    Radioactivity of acid-soluble organic phosphorus fraction (glycero-
phosphate-P[32]) in the serum of rats injected with glycerophophate de-
creased very rapidly, and rats in both groups were equal in the amount
of inorganic phosphate-P[32] in the serum at all times observed.   These
observations agree with those in the rabbits and indicate very rapid hy-
droysis of glycerophosphate in the blood serum.

    The sum of the activity of both incrganic and acid-soluble organic
phosphorus fractions in the serum of the group administered glycero-
phosphate-P[32] are significantly higher at 3 and 5 minutes after the in-
jection than those of the group injected with inorganic phosphate-P[32].

Therefore, it is seen that glycerophosphate is somewhat less permeable than inorganic phosphate.

The distribution of radiophosphorus in lower incisor, incior pulp, skull bone, liver, kidney, skeletal muscle, and diaphysis of femoral bone was also measured in both groups of the rats. The measurements are summarized in Table III.

The data of experiment 5 in Table III are not consistent with other experiments. In the experiments with 5-minute group, with the ex-
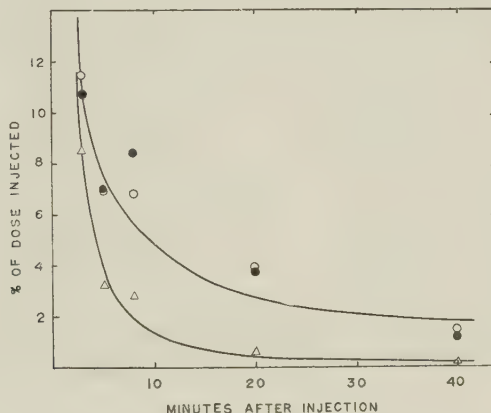

FIG. 1. Disappearance and hydrolysis of glycerophosphate-P^{32} in serum of rat compared to inorganic phosphate-P^{32}.

Rats were given intravenously 0.6 to 6 μg. of labeled phosphorus as inorganic phosphate or glycerophosphate. The values on ordinate are radioactivity in total serum as per cent of dose injected. ● ; Inorganic phosphate-P^{32} in the group injected with glycerophosphate-P^{32}, △ ; Acid-soluble organic phosphate-P^{32} in the group injected with glycerophosphate-P^{32}, ○ ; Radiophosphorus in the group injected with inorganic phosphate-P^{32}.

ception of experiment 5, there is no significant difference in radioactivity measurements on skull bone, serum inorganic phsphate fraction, and the kidney, between the groups injected with labeled inorganic phosphate and those with labeled glycerophosphate. However, in all the measurements for experiment 5, glycerophosphate group largely exceeded inorganic phosphate group. Because of the difficulty of getting a strictly defined portion in the samples of skull bone and femur diaphysis, the values in Table III are given as per 100 mg. of wet tissues. Therfcre,

Y. ITO, S. TSURUFUJI AND Y. HIRAMATSU

TABLE III

Distribution of Radiophosphorus Originating from Glycerophosphate-P³² in Comparison with Inorganic Phosphate-P³²

Rats were given intravenously 1 to 5 μc. of glycerophosphate or inorganic phosphate labeled with P^{32} accompanying 0.6 to 6 μg. of carrier phosphorus.

Expt. No.	Litter No.	No. of rats	Chemical form of injected radio-P	Body weight	Time of sacrifice	Total serum[1] Acid-sol. organic	Total serum[1] Inorganic	Lower incisor[2]	Lower incisor pulp[3]	Skull bone[4] per 100 mg.	Kidney (left)	Femur diaphysis per 100 mg.	Liver	Muscle per 100 mg.
				g.	mins.									
1	—	3	Glycero-P	147	40	0.11	1.49	0.25[5]	—	—	—	—	—	—
		3	Inorg.-P	151	40		1.21	0.19[5]	—	—	—	—	—	—
2	I	2	Glycero-P	277	20	0.64	4.03	0.26[5]	—	—	—	—	—	—
		2	Inorg.-P	257	20		4.29	0.15[5]	—	—	—	—	—	—
3	II	2	Glycero-P	235	20	0.70	3.52	0.31[5]	—	—	—	—	—	—
		2	Inorg.-P	246	20		3.70	0.21[5]	—	—	—	—	—	—
4	—	4	Glycero-P	218	5	2.82	6.69	0.108	0.024	0.129	—	—	—	—
		4	Inorg.-P	215	5		7.36	0.085	0.018	0.129	—	—	—	—
5	—	4	Glycero-P	248	5	3.84	7.30	0.080	0.016	0.102	1.77	—	—	—
		4	Inorg.-P	246	5		4.82	0.049	0.010	0.058	0.99	—	—	—
6	III	2	Glycero-P	199	5	2.67	7.31	0.135	0.014	0.148	2.35	0.098	6.63	0.239
		2	Inorg.-P	192	5		7.08	0.098	0.010	0.151	1.83	0.116	8.22	0.287
7	IV	3	Glycero-P	163	5	2.17	6.37	0.215	0.023	0.175	2.04	0.113	7.94	0.328
		3	Inorg.-P	165	5		5.94	0.187	0.020	0.169	2.03	0.131	9.50	0.354

Recovery of P^{32} as per cent of dose injected

[1] The values were calculated on the assumption that the total volume of the serum of a rat is 2.5 ml. per 100 g. body weight (7). [2] Mean value of two lower incisors. [3] Bilateral. [4] A defined portion of combined frontal and parietal bones. [5] Containing dental pulp.

it is natural that the values should vary in correlation with body weight of animals.   Distinct rectilinear relationship between the uptake of activity by the skull bone and the body weight of animals is actually shown in Fig. 2.   No difference between glycerophosphate and inorganic phsphate groups is found in Fig. 2 either, with the exception that the values of inorganic phosphate group of experiment 5 are alone markedly lower.   The cause of the discrepancy between experiment 5 and others is obscure, but some procedural mistake or unknown latent effect may be responsible.   Therefore, the authors will discard the data on experiment 5 in subsequent discussions.
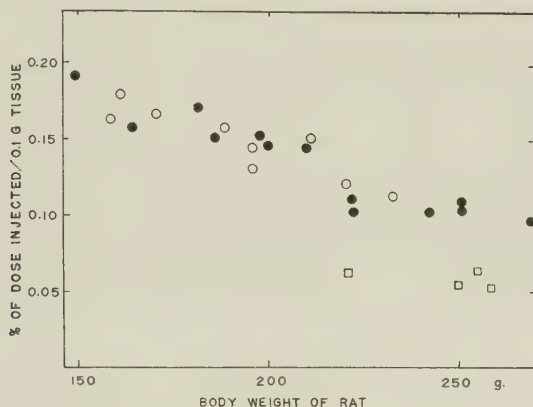

FIG. 2.   Correlation of body weight of rat to uptake of radio-phosphorus by skull bone.

Rats were injected intravenously with 0.6 to 6 μg. of labeled phosphorus as inorganic phosphate or glycerophosphate, and sacrificed 5 minutes after the injection.   The values on ordinate are radioactivity in 0.1 g. of skull bone as per cent of dose injected.   ● ; Rats injected with glycerophosphate-P[32],   ○ ; Rates injcted with inorganic phos-phote-P[32],   □ ; Rats injected with inorganic phosphate-P[32] in Experiment 5 in Table III.   The values are somewhat extraordinarily lower.

Most striking feature in Table III is that the values on incisor teeth and incisor pulps of the glycerophosphate groups were usually, but not always statistically significantly, higher than those of the inorganic phosphate groups in all the experiments, though the values in the serum, liver, muscle, and diaphysis of femoral bone of the former groups were

somewhat lower.   Statistical analysis indicates that the rats in glycero-
phosphate groups of the 5-minute experiment took up much more radio-
phosphorus in lower incisor ($p < 0.025$) and in lower incisor pulp ($p <$ 0.01).

That the difference in the uptake of radiophosphorus by incisor teeth
between the two groups is statistically not significant in the 40- and 20-
minute experiments is probably due to rapid hydrolysis of glycerophos-
phate-P^{32} *in vivo* and to the too small a number of rats for the confir-
mation to be admitted statistically.

Since it has been suggested that glycerophosphate is less permeable
than inorganic phosphate, more rapid uptake of radiophosphorus of in-
organic form by soft tissues would be natural.   On the contrary, how-
ever, incisor teeth and, in spite of soft tissues, incisor pulps of the glycero-
phosphate group took up more radiophosphorus than those of the inorganic
phosphate group in the 5-minute experiments.   Since incisor of the rat
is a most actively calcifying organ, the results would be closely related
to the mechanism of calcification.

From the time when R o b i s o n proposed a classical theory on the
role of phosphoric ester and alkaline phosphatase in the process of calcifi-
cation, voluminous reports have appeared on this subject.   G u t m a n
and Y u (*8*) related glycogenolytic phosphorylation process to the me-
chanism of calcification.   C a r t i e r and P i c a r d (*9*) attached a great
importance on ATP and ATP-ase.   On the contrary N e u m a n *et al.*
(*10*) considered phosphoric ester to be an interfering factor for the calcifi-
cation process.   Whether ester phosphate is an impotant source of the
phosphate for caclification or merely adsorbed on mineral surfaces as
interpreted by N e u m a n *et al.* (*10*), radiophosphorus originated from
labeled glycerophosphate would be largely retained in actively calcifying
surfaces of mineralized tissues.   The present data on incisor teeth alone
do not help in deciding which is a more appropriate explanation for the
role of ester phosphate.   On the other hand, the rats in glycerophosphate
group did not surpass inorganic phosphate group in the uptake of radio-
phosphorus by the skull bone and the diaphysis of femoral bone.   In
these bone tissues calcification process is not so active, and therefore,
the discrepancy between these bone tissues and the incisors   seems to indi-
cate that phosphoric ester is essentially related to the calcifying process
itself.   Moreover, a higher accumulation of radiophosphorus in the
dental pulp of vigorously calcifying incisor teeth of rat suggests an im-
portant role of phosphoric ester in the mechanism of calcification.

SUMMARY

A mixture of α-and β-glycerophosphate labeled with F^{32} with high specific activity was synthesized and administered intravenously to experimental animals in order to investigate their metablism, distribution, and incorporation into calcified tissues in comparison with inorganic phosphate-P^{32}.

1.   Glycerophosphate injected intravenously is rapidly hydrolysed and resulting inorganic phosphate is liberated into the blood stream and excreted into urine.   As specific activity of inorganic phosphate in the urine was higher than those of the serum, it is found that phosphatase in the kidneys releases inorganic phosphate from phosphate ester into urine.

2.   Determinations of radioactive phosphorus remaining in the serum indicates that inorganic phosphate is more diffusible through cell membranes than glycerophosphate.

3.   Actively growing incisor teeth of a rat and dental pulps of the incisor took up more radiophosphorus from glycerophosphate-P^{32} than inorganic phosphate, contrary to the results for other tissues examined. This indicates that phosphorus in glycerophosphate has a specific affinity to actively calcifying tissues and suggests an important role of ester phosphate in the mechanism of calcification.

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LETTERS TO THE EDITORS

THE ACTION OF LEUCINE AMINOPEPTIDASE ON PROTAMINES*

Sirs:

For the purpose of elucidating the specificity of leucine aminopeptidase (LAPase) and the N-terminal structures of protamines, the sulfates of clupeine and salmine were digested as follows with the purified LAPase prepared from swine kidney according to the method of S m i t h *et al.* (*1*).

A solution of 3.0 mg. of LAPase (treated with DFP) in 1.5 ml. of 0.005 M veronal buffer of pH 8.5, containing 0.005 M MgCl$_2$, was preincubated at 37° for one hour, then a solution of the clupeine sulfate** (15 mg. in 2 ml. of 0.002$_5$ M veronal buffer of pH 8.5, containing 0.002 M MgCl$_2$ and 0.5 M LiCl) was added to the above and the incubation was continued thereafter (clupeine sulfate: LAPase=200:1, mole/mole). 0.5 ml. each of the reaction mixture was taken out at various periods of time, and was poured into a column (0.6 × 5 cm.) of Amberlite XE-64 bufferized at pH 8.0 by 1 N NaCl-0.1 M Na$_2$B$_4$O$_7$. A group of monoamino acids and arginine which have been liberated during the digestion were eluted separately from the column, and the amount of the total monoamino acids and arginine was determined by the ninhydrin method and S a k a g u c h i's method, respectively. The clupeine core remained in the upper part of the column. For the determination of individual monoamino acids, two methods were mainly used, one by dinitrophenylation and the other by ninhydrin colorimetric procedure after one-dimensional paper chromatography with *n*-butanol-formic acid-water (75:15:10, *v/v*) according to the method of G i r i *et al.* (*3*). The result obtained was shown in Fig. 1.

As previously reported (*5*), it has been found, that the specimen of the clupeine used in this experiment consists of a mixture of equal quanti-

ties of peptide chains containing alanine and proline in the N-terminal, respectively. As is seen from the figure, however, the liberation of proline in the reaction mixture after 24 hours was less than 30 per cent of the amount which should be calculated in the case of complete liberation of the N-terminal proline (0.5 mole) based on the molecular weight of 7,000 for the clupeine sulfate. Therefore, it can be considered that two residues each of alanine and serine, and eight residues of arginine were liberated mainly from one mole of the alanyl chain in 24 hours.
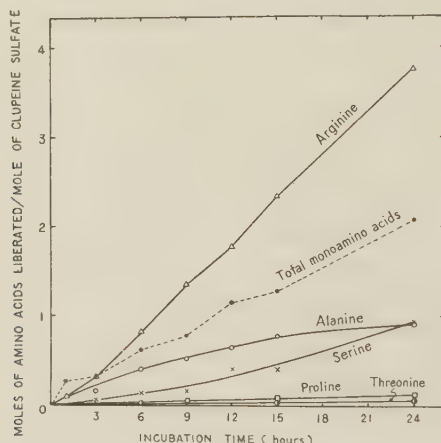

Fig. 1.   Rate of liberation of amino acids by the action of leucine aminopeptidase on clupeine sulfate.

Enzyme : Substrate=1:200, mole/mole, pH 8.5, at 37°. The molecular weight of clupeine sulfate was taken for 7,000. The determination of individual monoamino acids at 1, 3, 6, 12 and 24 hours was made by G i r i's method (3), that at 9 and 15 hours by L e v y's DNP-method, and only that of proline at 24 hours by C h i n a r d's method (4).

Furthermore, it is most probable that the serine residue occupies the fourth position from the N-terminus of the alanyl chain on the ground of the following three reasons : (a) At each reaction time (9, 12, 15 and 24 hours), the molar ratio of Arg/(Ala+Ser) which were liberated was practically 2.0 or slightly less. (b) More than one residue of serine has already been released (after about 16 hour incubation) prior to the liberation of two alanine residues (24 hours) from one mole of the alanyl chain. (c) Ala·Arg·Arg as the N-terminal peptide has already been

shown to be isolated from this clupeine preparation (5).

As to the salmine sulfate,* it was digested under the same condition as in the case of the clupeine, and all the amino acids liberated were determined by the dinitrophenylation method. In this case, however, approximately one residue of arginine and only a small fraction of proline and serine residues have been liberated in 24 hours per molecule of the salmine sulfate, assuming the molecular weight to be 7,000. From the estimation of the ratio (R) (5) of the optical density at 390 mμ to that at 360 mμ of the DNP-derivatives of the LAPase treated salmine in the reaction mixture, it was found that the proline residue still occupies the greater part of the N-terminal position of the protein at each reaction period of 3, 10, 20 and 24 hours.

From these results obtained with clupeine and salmine, it can be said about the specificity of the LAPase that the DFP treated enzyme shows none of an endopeptidase action, but releases amino acids successively from the N-terminal site of the protamine molecule, whereas it releases proline in the N-terminus only quite slowly.

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Institute of Science and Technology   TOSHIO ANDO
University of Tokyo, Tokyo   YUTAKA NAGAI
                                                       HIDEKO FUJIOKA
                    (Received for publication, September 4, 1957)

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* The less soluble fraction prepared from the fish material (*Oncorhynchus keta*) obtained at Chitose, Hokkaido, Japan, on Oct. 2, 1950 (2).

NOTES

THE ION EXCHANGE CHROMATOGRAPHIC SEPARATION OF THIAMINE PHOSPHATES AND THE OCCURRENCE OF THIAMINE POLYPHOSPHATES IN THE SYNTHETIC PREPARATION*

Siliprandi and Siliprandi (1) reported the chromatographic separation of thiamine and its mono-, di-, triphosphate by using the weak cation-exchanger, Amberlite XE-64. We, applying this method to the yeast, liver and rice bran extracts, found that the " TTP "** fraction obtained exerted much more thiamine activity against several thiamine-requiring microorganisms*** than that expected from the value of the thiochrome determination (2, 3). Further studies elucidated that this ion exchange chromatography with XE-64 was quite insufficient for the individual separation of the thiamine derivatives which were more acidic than TTP. Therefore, it became necessary to establish a different chromatographic system which enabled the complete separation of them.

The crude " TTP " fraction applied for the chromatography was prepared by the Siliprandi's XE-64 chromatography of thiamine phosphates synthesized according to the modified procedure of Viscontini et al. (4). The satisfactory chromatography have been achieved on columns of the strong anion exchanger, Dowex 1, X-2 or X-10, formate form by using the gradient elution technique (5). The principle was essentially same as that in the report of Hurlbert et al. (6). The details of the experimental conditions were described in the footnotes of Figures and Table. The results showed that in the crude " TTP " fraction there existed several thiamine polyphosphates besides TTP (Fig. 1). Analytical data (Table) clearly demonstrate that the peaks 2, 3 and 4 are tri-, tetra-, and pentaphosphate of thiamine, respec-

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* The main part of this paper was reported in the 8th annual meeting of Japan Vitamin Society (Fukuoka, 8–10, May, 1956) (3) and in the 92th meeting of Vitamin B research committee, Japan (Osaka, 2, March 1957) (*Vitamins*, **12**, 356, 1957).

** The following abbreviations are used. Thiamine mono-, di- and triphosphate are TMP, TDP and TTP.

*** *Lactobacillus fermentii*, ATCC 9338 : *Lactobacillus fructosis nov. sp.* (*8*).

tively.   Peak 1 was identified with TDP by comparing with the standard sample.   Peaks 5 and 6 could not be tested due to their small amounts. The overall yields attained to 75–85 per cent as determined by thio-
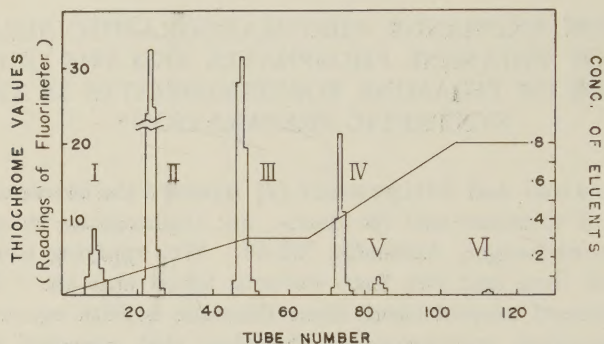

Fig. 1. Chromatography of thiamine phosphates with Dowex 1 column. Dowex 1, X-10 (formate form, 200–400 mesh) 1×23 cm. Flow rate, 0.3 ml./minute. 7.5 ml. one fraction. Sample, the crude "TTP" fraction, 3 mg.   The line shows the ammonium formate (pH 4.5) concentration of the eluent (M).

TABLE   I

Molar Ratios of Total, Acid-labile Phosphorous and Thiamine

Sample		Phosphorus		Thiamine	Suggested compound
		total	acid-labile		
Fig. 1	Peak 2	3.00	2.07	1.01	thiamin triphosphate
	Peak 3	4.00	2.95	1.02	thiamine tetraphosphate
	Peak 4	5.00	4.08	0.99	thiamine pentaphosphate
Fig. 2	Spot 1	3.00	1.75	1.01	thiamine triphosphate
	Spot 2	4.00	3.16	0.99	thiamine tetraphosphate

Thiamines was determined by thiochrome method (*10*) by using Kotaki fluorometer, Type UM. Phosphorous was determined by Fiske, Subbarow method after wet ashing with sulfuric acid or heating in 1 N HCl at 100° for 7 minutes.

chrome method (*10*). Rechromatography of the material of peak 2 or 3 of Fig. 1 gave a main peak in the same position as in Fig. 1, re-

spectively, with only small peak (less than 5 per cent) of TDP or TTP. The latters were possibly produced due to the decompositions during lyophilization and storage.

The paper electrophoretic studies further confirmed these results. Fig. 2 and Table showed that the original sample contained thiamine tetra- and pentaphosphate which moved faster than TTP towards the anode. Both electrophoretic and chromatographic data were well co-incident each other.

From these results it was shown that this chromatographic system with Dowex 1 was very favourable for the separation of the acidic thiamine derivatives which could not be separated each other by the XE-64 system. In addition, it was demonstrated that the synthetic thiamine phosphates preparation contained tetra- and pentaphosphates, at least. Just before finishing this manuscript, a report of H. K. Kiessling
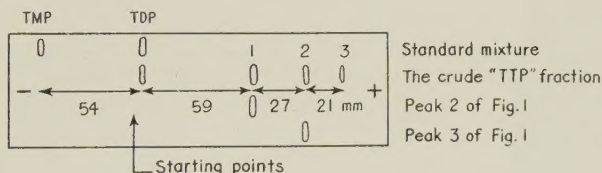

Fig. 2. Paper electrophoresis of thiamine phosphates.
0.1 M acetate buffer, pH 4.5 ; 150 volt/cm., 15 minutes ; Cooled in n-hexan (9) ; Paper, " Toyo ", No. 5B, 6 × 24 cm. Sprayed with 0.1 per cent ferricyanide, 1 per cent NaOH solution after drying.

(7) was published, demonstrating the presence of thiamine polyphosphates in a similar synthetic preparation by means of the paper partition chromatography.

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Research Laboratories,   SUZUOKI-ZIRO
Takeda Pharmaceutical Ind., Ltd.   MASAHIKO YONEDA
Osaka, Japan   MASATAKE HORI